THE EFFECT OF OZONE INHALATION ON THE FREQUENCY OF CHROMOSOME ABERRATIONS OBSERVED IN IRRADIATED CHINESE HAMSTERS

By

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THE EFFECT OF OZONE INHALATION ON THE FREQUENCY OF CHROMOSOME ABERRATIONS OBSERVED IN IRRADIATED CHINESE HAMSTERS

By

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Should presently permissible human exposure levels for ionizing radiation and for respirable ozone be reduced when exposure to both agents takes place, especially simultaneously? This particular combination of noxious agents warranted consideration for three reasons.

1. In many of its actions on biological materials, ozone mimicked the effects of ionizing radiation; this included the ability to produce chromosome aberrations in human cells _in vitro_;
2. The direct effects of inhaled ozone did not appear restricted to the pulmonary system, but were widely distributed throughout the body;
3. Situations existed in which people were exposed to these two agents simultaneously; there were also indications that more people would be similarly exposed in the future.

Chromosome aberrations produced in circulating blood lymphocytes were the indicator of biological damage. Effects in these cells would be a qualitative indicator of similar damage to other cells throughout the test animal. Exposure-adjusted break frequencies served as the quantitative measure of effect. Adult female Chinese hamsters,

Cricetulus griseus ($2n = 22$), were exposed, in groups of four, to x-radiation (118 keV effective, 70 cm FSD, 230 rad dose), to ozone (0.2 ppm, UV generated), or to both simultaneously. All exposures were of 5 hr duration. There were two groups per treatment and two additional groups, one serving as a control and the other exposed to 330 rad. A total of 50 animals were utilized in the investigation, including 18 in preliminary experiments.

Blood samples (0.2 ml) were obtained by orbital bleeding. Following plasma removal, the cells were cultured in an enriched media for 3 days at 37°C in a humidified 5 percent CO_2 environment with pokeweed as the mitogen (lymphocyte transformation and division stimulant). Division was arrested in metaphase with colchicine. Following hypotonic KCl treatment to swell the cells, they were fixed with a 3:1 methanol-acetic acid solution, transferred to a slide, and rapidly dried by ignition. The slides were stained with Giemsa and scanned using bright-field microscopy. Spreads were photographed on 35 mm film and projected onto a screen for analysis (with subsequent microscope rechecks of spreads exhibiting breaks). The aberrations scored were deletions, dicentrics, and rings.

The following were the principal results of this investigation:

1. Radiation resulted in an exposure-adjusted break frequency of $5.51 \times 10^{-4} \frac{\text{breaks}}{\text{cell-rad}}$ for cells withdrawn 2 weeks after exposure. This value appeared reasonable in comparison to available information on in vivo exposure of human lymphocytes and Chinese hamster bone marrow cells. Successful lymphocyte cultures could not be obtained until 2 weeks had elapsed. Break frequency appeared to vary linearly with dose in the region 230-330 rad.

2. Ozone resulted in an exposure-adjusted break frequency of $1.67 \times 10^{-3} \frac{\text{breaks}}{\text{cell-(ppm-min)}}$, agreeing well with the value expected from *in vitro* exposure of human cells. There was no apparent decrease in break frequency with time for 2 weeks post-treatment.

3. Animals exposed to the two agents simultaneously exhibited $>$70 percent of the total number of breaks anticipated assuming additive actions. Expected contributions from ozone and from radiation were nearly equal. There was, however, approximately an 18 percent (15 percent) chance that all the breaks observed resulted from the radiation (ozone) exposure alone.

Presently permitted human ozone exposures (up to 0.1 ppm, 4 $\frac{\text{ppm-hr}}{\text{week}}$) would be expected to result in break frequencies that are orders of magnitude greater than those resulting from permitted human radiation exposures if the results of this experimental animal study were directly extrapolated to the human case. Consideration of combined ozone plus radiation environments is overshadowed by the importance of ozone environments alone as long as permitted ozone exposure levels remain at their present values.

CHAPTER I

INTRODUCTION

Man has become increasingly concerned about his surroundings as they relate to his health and well-being. The complex environments which he has created through technology make it necessary for him to consider how various agents and conditions of these environments affect him and how they can interact with regard to his health. Ozone and ionizing radiation are two agents which have been extensively investigated individually and whose combined actions have been under study.

Ozone is a highly reactive three-atom allotrope of oxygen. It is present in the atmosphere as a natural constituent and is produced artifically both intentionally and as an unwanted byproduct of various operations. The biological effects of ozone on man and animals have received much consideration. Many studies have dealt with the respiratory system effects (1-46), as inhalation is the principal path of entry. It was believed that the action of ozone was restricted to the respiratory system, for this is where its effects are most noticeable, particularly at concentrations of 0.3 parts per million (ppm) by volume and higher (29).

Now there is evidence that the action of ozone is more widespread. Its effects on a wide range of other body functions and systems have been considered (47-76) as have its effects when present in combination with other agents including other gases, aerosols, mists, and bacteria (26, 62, 66, 77-81). A number of general reviews of these biological effects of ozone on man and animals are available (54, 82-85). The present occupational exposure limit for ozone has been set at 0.1 ppm.

Ionizing radiation is probably one of the most-studied physical insults to which man has been exposed. Background sources of irradiation are cosmic rays and emissions from naturally occurring radioactive materials; technological sources include x-ray generators, artifically produced radioactive materials, nuclear reactors and detonations, and particle accelerators. The effects of ionizing radiation on biological systems are extremely broad in range. This results from the penetrating ability of many of the emissions. One of the most comprehensive publications on this subject is the Report of the United Nations Scientific Committee on the Effects of Atomic Radiation (86). This report covers fundamental radiobiology, hereditary and somatic effects of radiation, sources of irradiation, and comparison of doses and estimates of risks, and includes nearly 3,000 references. A later report in 1964 having the same title (87) acts as a supplement to the comprehensive 1962 work. There is, additionally, an annotated indexed bibliography on the biological effects of ionizing radiation that covers world literature for the time period 1898-1957 and includes almost 13,000 entries (88). A supplement to this extensive work covers 1958-1960 and contains nearly 12,000 more entries (89).

The Radiomimetic Nature of Ozone

The property that incited the considerable interest in ozone, alone and in conjunction with other agents, is its large electronegative potential, -2.07 V, ($O_2 + H_2O \rightarrow O_3 + 2H^+ + 2e$). This value is only slightly exceeded by fluorine's, -2.1 V ($2F^- + H_2O \rightarrow F_2O + 2H^+ + 4e$) (90). Thus, ozone is an extremely powerful oxidizing agent. It has been proposed that the oxidizing action of ozone is via a free radical mechanism (16, 91).

Ionizing radiation, when interacting with biological systems, also acts as a powerful oxidizing agent, this through the action of free radicals formed by the dissociation of water (92). The processes involved in the initial interactions of ionizing radiation with water are (93, 94):

Exitation

$$HOH \xrightarrow[\text{radiation}]{\text{ionizing}} H\cdot + \cdot OH \tag{1}$$

Ionization

$$HOH \xrightarrow[\text{radiation}]{\text{ionizing}} (HOH)^+ + e \tag{2}$$

followed by

$$H^+ + e \rightarrow H\cdot \tag{3}$$

or

$$H_2O + e \rightarrow H\cdot + \cdot OH^- \tag{4}$$

and possibly

$$(HOH)^+ \rightarrow H^+ + \cdot OH \tag{5}$$

Combination results in the following postulated reactions (93, 94):

$$H\cdot + \cdot OH \rightarrow HOH \tag{6}$$

$$H\cdot + H\cdot \rightarrow H_2 \tag{7}$$

$$\cdot OH + \cdot OH \rightarrow H_2O_2 \tag{8}$$

or

$$\cdot OH + \cdot OH \rightarrow H_2O + \cdot O \tag{9}$$

and

$$\cdot O + \cdot O \rightarrow O_2 \tag{10}$$

and if dissolved oxygen is present

$$\cdot OH + \cdot OH + O_2 \rightarrow H_2O_2 + \cdot O + \cdot O \tag{11}$$

$$\cdot H + O_2 \rightarrow \cdot O_2H \tag{12}$$

and possibly

$$\cdot O_2H + \cdot O_2H \rightarrow H_2O_2 + O_2 \text{ ....} (13)$$

Of the above, 6 and 7 remove reducing agents while 8-13 produce oxidizing agents. These oxidizing agents or the OH radical itself (as a highly reactive electron acceptor, $OH^- \rightarrow \cdot OH + e$ -3.7 eV) act to oxidize inorganic ions and/or organic compounds that are present in the aqueous biological system (93).

Thus, through oxidizing processes, ozone and ionizing radiation might be expected to act similarly on biological materials that are exposed to them. This raises the question of whether tissues beyond the pulmonary system are exposed to the action of ozone when ozone is being inhaled. To put it another way, will ozone inhalation result in direct extrapulmonary effects as opposed to secondary systemic reactions resulting from pulmonary involvement? If other tissues are directly affected, then the similarity between exposure to these two agents, ionizing radiation and ozone, will be even more striking. For just as an experimental animal in a radiation field would be expected to have effects of the exposure throughout its body, an animal in an ozone environment would similarly experience widespread direct effects of its exposure through inhalation.

There is a variety of observations on the actions of ozone that demonstrate direct extrapulmonary effects of ozone inhalation. Some of these effects are similar to those seen after exposure to ionizing radiation. These latter observations have resulted in ozone being widely considered as a "radiomimetic" agent. The following is a listing and discussion of experiments demonstrating direct extrapulmonary and/or radiomimetic effects of ozone exposure:

1. Cutaneous oxygen consumption in a digit of man has been shown to be strongly affected by short-term inhalation of moderate ozone concentrations (95). The technique involved the sudden occlusion of digital circulation and the estimation of the ensuing deoxygenation of oxyhemoglobin photoelectrically. Stagnant intracapillary deoxygenation normally proceeded logarithmically to complete dissociation in approximately 10 min. However, the dissociation immediately following the breathing of 1 ppm ozone for 10 min only reached the 50 percent level 10 min after occlusion and appeared to level off. This phenomenon, interpreted as a reversible intoxication of final heme-enzyme groups in the redox chain, was also evident following the breathing of pure oxygen or irradiation of the finger with ultraviolet light. In the case of ozone, normal deoxygenation was evident if cysteamine was introduced into the skin immediately after ozone inhalation. The interpretation of these results was that ozone reacted with the linings of the airways, and the oxidizing products of this interaction were distributed by the circulation of the blood. Cysteamine, as it did with ionizing radiation, provided chemical protection presumably by inactivating oxidizing agents through a radical scavenging mechanism. Thus, extrapulmonary oxidizing action was inferred.

2. The acute toxic action of inhaled ozone has been reduced by the simultaneous injection or intraperitoneal injection of compounds that furnish -SH or -SS- bonds (96). Compounds with -S bonds proved ineffective. As was mentioned previously, these same chemicals provided protection from exposure to ionizing

radiation (86). This further suggests a similarity of action for ozone and radiation.

3. The effects of ozone inhalation on the visual acuity of man have been investigated (97). Levels of 0.2-0.5 ppm inhaled for 3 hr or two 3 hr periods with 1 hr rest between resulted in (a) decrease in visual acuity for dark adaptation and middle vision ranges, (b) increases in peripheral vision, and (c) change in the balance of most extraocular muscles. The effect, while not radiomimetic, demonstrates direct extrapulmonary action of ozone.

4. In animals, including man, red cells start circulating as flat discs and end up as spherocytes. The process can be greatly accelerated by x-irradiation in vitro. If ozonized air is inhaled before the blood is withdrawn, this process is further accelerated (98). Distinct effects were seen in man with ozone exposures as low as 0.25 ppm for 30 min. For example, for blood drawn 1 hr after this degree of ozone exposure, 7,000 R resulted in 55 percent of the red cells being spheroid compared to 21 percent for non-ozone-exposed controls. Sphering tendency acceleration by ozone was a reversible reaction, as the amount of sphering seen decreased with time after ozone administration. The experiment demonstrated a distinctly radiosensitizing effect of ozone inhalation and showed ozone action on the blood system.

5. It has been demonstrated that the inhalation of ozone can result in structural damage of postmitotic nuclei in myocardial fibers of adult rabbits and mice (98). Exposure to 0.2 ppm, 5 hr/day for 3 weeks,resulted in rupture of nuclear envelopes and extrusion of contents which are never observed in the nuclei

of normal fibers. This type of damage is also seen in patients following therapeutic radiation exposure and in irradiated tumor cells. Here, extrapulmonary tissue damage which mimics that seen from radiation exposure was shown.

6. Another indication of the deep and radiomimetic effects of ozone was its effects on the offspring of exposed mice (98). The exposures were to 0.1 or 0.2 ppm, 7 hr/day, for 3 weeks. For inbred grey mice, litter sizes were normal, but 3 week neonatal mortality was 6.8 percent (0.1 ppm) and 7.5 percent (0.2 ppm) compared to 1.6 percent for controls. For the second litters from parents exposed to 0.1 ppm, 4.9 percent mortality was seen against 1.9 percent for controls. For highly inbred C57 black mice, exposure to 0.2 ppm under the same routine resulted in normal size litters but 34 percent neonatal mortality compared to 9 percent for controls. Here, then, we have evidence of a lingering observable effect on gonadal material not unlike effects of ionizing radiation (86).

To these experiments which demonstrate widespread direct extrapulmonary ozone effects along with the oxidizing and radiomimetic nature of ozone action can be added another group of studies involving the exposure of animals to ozone in conjunction with lethal doses of ionizing radiation. In these, the effects on radiosensitivity of preexposure to ozone were investigated. These experiments are described in Table 1.

In each case, ozone inhalation had an effect on mortality. The radiation doses used were approximately $LD_{50/30}$'s (i.e., doses that should result in the deaths of 50 percent of the animals exposed within

TABLE 1

EFFECT OF OZONE INHALATION ON MORTALITY RESULTING FROM RADIATION EXPOSURE

[O$_3$], ppm	Duration, hr	Relation of O$_3$ Exposure to Radiation Exposure	Radiation Type	Radiation Exposure, R	Animal	Results	Reference
0.2	1	O$_3$ immediately prior to radiation	x-ray	675	mice	30% survival ($\geq$18d) compared to 60% for x-ray alone	99, 101
0.2	7.5	O$_3$ immediately prior	x-ray	775	mice	70% survival ($\geq$25d) compared to 0% for x-ray alone	99, 101
0.2	24	O$_3$ immediately prior	x-ray	775	mice	50% survival ($\geq$25d) compared to 0% for x-ray alone	99, 101
0.2	1	O$_3$ immediately prior	x-ray	675	mice (4 months)	18% survival ($\geq$16d) compared to 65% for x-ray alone	100, 101
2	0.25, 0.5	O$_3$ 1 day prior	x-ray (230kV)	550	mice (adult albino)	100% survival ($\geq$30d) compared to 40% for x-ray alone	102
1	1	O$_3$ 1 day prior	x-ray (230kV)	550	mice (adult albino)	100% survival ($\geq$30d) compared to 40% for x-ray alone	102
1	1	O$_3$ 10 days prior	x-ray (230kV)	800	mice (adult albino)	60% survival ($\geq$30d) compared to 0% for x-ray alone	102

TABLE 1
CONTINUED

$[O_3]$, ppm	Duration, hr	Relation of O_3 Exposure to Radiation Exposure	Radiation Type	Radiation Exposure, R	Animal	Results	Reference
0.05 or 0.1	1	O_3 immediately prior	x-ray	500 to 675	mice (grey, 3 & 4 months)	decreased survival compared to x-ray alone	103
0.025, 0.25, 2.5, 15	1	O_3 prior to radiation	Co-60	650	rats	40, 20, 33, and 0% survival ($\geq$30d) compared to 47% for radiation alone	104
1	1	O_3 1 day prior	x-ray	LD_{50} [approx. 550]	mice	O_3 gave radiation dose reduction factor of 1.3 (i.e., O_3 gave tolerance	105
(1 to 2?)	($\leq$1 hr?)	($O_3 \geq$1 day prior ?)	Co-60	lethal [approx. 550]	(mice?)	no protection	91

30 days) or greater. The ozone exposures were all considerably below lethal levels. (For mice, exposure to 6 ppm for 4 hr results in 50 percent mortality. Rats are even more resistant (84).) Most were below the approximately 6 ppm-hr at which edema and lympocyte migration into alveolar spaces begin, i.e., where lung histology changes (68). Some were even below the 0.7 ppm-hr where lung function temporarily changes during exposure (27). (For short term single exposures, the product of ozone concentration and exposure time produces a constant toxicological response (84).) Since little if any effect on pulmonary structure or function could be expected once the ozone exposures ceased, the effect on mortality of subsequently administered lethal doses of radiation is an additional demonstration of the direct extrapulmonary action of inhaled ozone.

It should be noted in Table 1 that two phenomena are exhibited. The first, radiosensitization, acts to increase mortality. This is seen when the ozone exposure is of short-term duration (1 hr) and immediately prior to the radiation exposure. If the ozone exposure is of longer duration immediately prior to the radiation or of short duration but with a time lag of 1 day or more between ozone exposure and radiation exposure, the effect is to decrease mortality, radioprotection!

To interpret these apparently opposing observations, it should be recalled that both ozone and radiation have oxidative processes in the body as the basis of their action. With ozone as the radiosensitizer, the oxidation from it (on the premise that it does occur throughout the body and not just in the respiratory system) adds to the oxidation resulting from the radiation to bring about a level of damage greater than that from the radiation alone; hence increased mortality. To bolster this interpretation, it has been shown that small doses of ionizing radiation (≲65 R) immediately preceeding lethal ozone exposures increase the mortality observed over that from ozone alone (104). With ozone as the radioprotective agent, the interpretation is that the insult from the ozone, i.e., the oxidation from it, was sufficient in magnitude to bring about an antioxidant response by the body and to stimulate intracellular repair mechanisms before the radiation exposure began. Through this, some of the oxidizing action of the radiation was counteracted, resulting in a lower level of damage and decreased mortality.

This antioxidant response of the body is equally effective if the source of subsequent oxidant is an additional ozone exposure of lethal magnitude rather than radiation exposure (34). Likewise, pre-exposure to ionizing radiation rather than to ozone can initiate the presumably antioxidant response (106-108). Since this is the case, ozone is also radiomimetic in these experiments in which it acts as a radioprotective agent.

Thus, a variety of observations have been made which rather conclusively indicate that there are direct extrapulmonary effects of ozone inhalation and that ozone can be generally considered as a radiomimetic agent.

The Presence of Combined Ozone and Radiation Environments and the Problem of Setting Exposure Limits

That ozone and radiation appear to act similarly on biological materials is of practical interest from a health and safely point of view. Man attempts to provide safe and comfortable environs for himself by, among other things, limiting his exposures to various agents to values below damaging or irritating levels, or at least to values for which the levels or risks of damage are acceptable when weighed against the overall benefits associated with the exposures. In deciding on acceptable levels for a particular agent, information from human experience and from animal and/or human experimentation is utilized. If two or more agents are present, the general practice is to consider their actions as additive (unless there is information to the contrary) and to accordingly reduce the permitted exposure level for each of them. This has been the case when the agents have some similarity to one another, as mixtures of organic solvents or mixtures of radioactive materials or radioactive substances and external irradiation in combination. There has been little if any consideration, however, of cross-combinations including the one under discussion here, ozone and ionizing radiation.

It appears important to consider the action of the ozone-ionizing radiation combination, particularly, because of the radiomimetic nature of ozone previously discussed, because there are presently situations where people are exposed to both agents simultaneously, and because there are definite indications that more and more people will be so exposed in the near future.

There are two broad classifications into which ozone-radiation environments fall. In the first, the radiation field is the source of the ozone; for the second, the principal source of the ozone is not the radiation field but other technological generating means, or the ozone is present naturally. There are some ozone-radiation environments that are a combination of these two types.

The irradiation of oxygen, gaseous or liquid, results in ozone production and in some decomposition of the ozone so formed. Alpha, fast electron, photon, and neutron irradiation have been investigated (109-116). The yield of ozone in a closed system increases with dose (109, 114-116) until an equilibrium value as high as 2,000 ppm (115) is reached at very high doses of approximately 10^7 rad (114). The yield also appears to increase with dose rate (114, 115), although this point is disputed (113). As would be expected, with a flow-through system, the concentration is reduced but the total amount of ozone produced increases (116).

It follows, then, that an ozone plus radiation environment can be associated with nearly any high-intensity radiation field. Some examples are research accelerators and isotopic sources, electron beam irradiators (used in industrial polymerization as for paint curing and plywood glue hardening), large-scale isotopic food irradiators, high-radiation areas at nuclear reactors, industrial radiography devices, and medical therapeutic radiation units.

This problem of ozone production by radiation has been recognized and investigated at a number of facilities including high-level gamma installations (117-119) and electron accelerators (117, 120-123). The principal method of control is usually to provide ventilation adequate for keeping ozone concentrations down to acceptable levels. In connection with this dicussion of radiation as a producer of ozone, it is interesting to note that a number of experiments with mice, rats, and monkeys have demonstrated that animals detect radiation by olfactory system response to ozone produced in their nasal passages by the radiation (124-127).

Other technological production of ozone is by electrical arcs or corona discharges, silent electric discharges, and shortwave (<2450 Å) ultraviolet light (128, 129). It is found industrially at ozonizers for treatment of sewage, for water purification, and for control of molds and bacteria at cold storage plants; in conjunction with high voltage electrical apparatus such as at generators, accelerators, x-ray devices, and spectrographs; and with electronically operated office copy equipment, neon signs, electric motor brushes, quartz ultraviolet lamps, and inert gas shielded arc welding units (7, 84, 130). A technologically related source of ozone is its environmental production in smog by photochemical reactions involving air pollutants and naturally present ultraviolet light (131).

The potential for a combined radiation-ozone situation exists wherever people are exposed to radiation while in the vicinity of other technological ozone sources. An example of this type of an environment is a nuclear submarine. Here the radiation source is the nuclear reactor power plant of the vessel; the ozone can be produced both by the radiation fields associated with the reactor and by the extensive electrical and electronic equipment aboard. The potential for ozone production on these ships has been recognized (132), and monitoring equipment is available on them (133). Control of the ozone concentration is complicated by the atmospheric recycling required by the nature and mission of such vessels.

Another example of an environment with a combined ozone-radiation exposure potential is a spacecraft. Radiation exposure can result from galactic cosmic rays, Van Allen belt particles, solar flares, radioactive debris from atmospheric nuclear explosions, and on-board radiation sources (134). Ozone can result from operation of the space cabin equipment (135) or from irradiation of liquid and gaseous oxygen supplied on-board (136). Here, too, the ozone situation is complicated by the atmospheric recycling required. As with the nuclear submarine, the ozone production potential has been recognized, as demonstrated by NASA sponsored experiments dealing with ozone toxicity (77, 78).

Ozone is also produced naturally in the upper atmosphere by the action of solar ultraviolet radiation on oxygen present in the stratosphere. The vertical concentration profile peaks at about 29.0 km and decreases rapidly as height decreases; there is also variation with latitude and season (84, 137). Concentrations up to 15 ppm (by volume) have been reported; natural ozone levels at ground level at U. S. latitudes are estimated at 0.01 ppm or less.

Present-day jet aircraft cruising at 9.2-12.4 km are at the edge of the so-called ozonosphere, while the supersonic transports (SST's) cruising at 19.8-24.4 km will be near its center, where the ambient external ozone level is hazardous to life. As ambient air is used for cabin pressurization currently and will be used for SST's, ozone levels in present aircraft have been investigated (138-142) and rigorous ozone control (decomposition and monitoring) is being planned for the SST (137, 143-145). The studies of present craft have indicated that the external air is the principal source of the ozone present (141), although cockpit levels are somewhat higher, suggesting other (electrical) sources (142). Levels range up to 0.4 ppm. A maximum level of 0.2 ppm is proposed for the SST (137).

While passengers and crews of present-day jetliners experience some radiation exposure, primarily from cosmic radiation, people in SST's will have to contend with a higher radiation level. This is a result of cosmic radiation being more intense at higher altitudes (6x higher at 21.4 km that at 9.2 km), protons from solar flares basically not penetrating the atmosphere to altitudes <18.3 km, and radioactive debris from high-yield atmospheric nuclear testing being predominantly concentrated in the stratosphere (above 11.0 km to 30.5 km) (146, 147). Thus, while jetliners do present a radiation-ozone environment, the SST will probably involve greater radiation exposure with the ozone exposures comparable to those presently encountered.

We are therefore confronted with the practical problem of having people simultaneously exposed to ionizing radiation and ozone, along with the prospect of even more people being so exposed in the future. In view of the apparently radiometric nature of ozone, should the maximum levels of exposure permitted for each agent when considered as acting alone still apply in this dual exposure condition? Presently, the radiation and ozone exposure limits are applied individually. A quantitative measure of widespread damage throughout the body is needed that will serve equally well to evaluate ozone and radiation exposures. Such a measure would indicate whether the agents in a combined environment acted in an additive fashion, synergistically, or antagonistically in their damage production. Then a decision could be reached as to the adjustments, if any, that should be made in the individual exposure limits when a combined exposure occurs.

Chromosome Aberrations As an Indicator of Radiation and of Ozone Exposures

A brief introduction to chromosome organization and structure is presented here because it forms the basis for the experimental rationale.

Chromosomes are nuclear organelles. They are in the form of elongated threads (present in the nuclei of cells) between cell divisions, or in interphase. During division, they contract into short thick readily observable rods. Chemically, they are characterized as nucleoproteins having the polynucleic acid DNA (deoxyribonucleic acid) and two specific proteins as their main components. Functionally, through subdivisions called genes, they dictate the enzyme and protein production and hence the metabolic chemical reactions of the cells in which they occur. This information is transmitted from generation to generation in a cell line by duplication of each chromosome in the mother cell prior to division (mitosis) so that at division each daughter cell gets a complete complement of chromosomes and thereby a complete code for protein production. Through meiotic cell division, each sexual gamete receives one-half the characteristic number of chromosomes. When fertilization occurs, the normal number of chromosomes for cells of the particular species is restored.

While genes are remarkably stable, alterations do occur. A change in a gene that is accompanied by a visible change in the structure of the chromosome of which it is a part is called a chromosomal mutation; those gene changes that are not accompanied by visible chromosomal changes are called point mutations, or gene mutations. As genes control the metabolic reactions of the cell, gene changes can be highly detrimental (even lethal) or relatively insignificant.

A chromosomal mutation, or aberration, results when the two ends created by a single break in a chromosome fail to rejoin with one another through repair mechanisms. Rejoining can fail to occur or ends from two different breaks in the same or in separate chromosomes can join to produce a variety of aberrations. These aberrations often result in an unequal distribution of chromosomes between daughter cells, and this usually leads to cell death. If rejoining of the two ends created by a single break does occur, restitution may be at the morphological level only, and a point mutation due to DNA damage may appear (84). It is for these reasons that chromosome aberrations are considered as cellular alterations (damage) with long-term consequences.

There are a variety of agents which have the capacity to bring about gene changes, i.e., which are mutagenic. Ionizing radiation has long been recognized as one of these agents (93, 148). Its effectiveness is dependent on the magnitude of the dose (direct dependence), the cell type, and the stage of the cell cycle at the time of irradiation, but all living cells are susceptible to this action (86, 148).

Evidence has accumulated that ozone, too, is a general mutagenic agent. It has been observed to change the adsorption spectra of nucleic acids (149); to markedly modify the pyrimidine bases (thymine, cytosine, and uracil) in _E. coli_ nucleic acids (150); to produce specific mutants of _E. coli_ exposed in ozonated water (151); to result in various chromosome aberrations when tissue cultures of embryonic chick fibroblasts were exposed to gaseous ozone (152); to produce a high frequency of chromosome aberrations in the root meristems of _Vicia faba_ (153); and, finally to produce a number of different types of chromosome aberrations in cultured human cells exposed to gaseous ozone (154). For this last investigation, the frequency of aberrations seen in human cells was shown to depend directly on the magnitude of the ozone exposure. Comparison was made with the frequency of aberrations seen with exposure of the cells to x-radiation. For radiation, the yields also varied directly with the dose.

It appears that ozone, like ionizing radiation, could be expected to produce chromosome aberrations in all types of cells that are exposed to it. Because of the radiomimetic nature of ozone, this is not a surprising observation. Likewise, this action of ozone, production of chromosome aberrations, could be expected to occur throughout the body of an animal inhaling ozone in view of the direct extrapulmonary effects of ozone inhalation that have been demonstrated.

Therefore, it appears that the frequency of chromosome aberrations produced by exposure of an animal to ionizing radiation and/or to ozone can serve as the sought-after quantitative measure of widespread damage.

Blood Lymphocytes As the Test Cells

There are numerous technical difficulties associated with the production of mammalian chromosomal preparations. The usual histological techniques do not suffice, and squash preparations are not completely satisfactory, partly because of the small size of mammalian cells and their relatively large chromosome numbers (155). Since the early 1950's it has been possible to make suitable preparations from mammalian cells maintained in tissue cultures by employing a hypotonic pre-fixation treatment to swell mitotic cells (156). Unfortunately, this has little value for experiments involving whole animals. Similarly, good preparations have been made from tissue biopsy cells put into suspension and subjected to the same hypotonic pre-treatment procedure; a soft, easily dispersed tissue is required. Skin and bone marrow have been used, but sample collection is difficult, particularly when repetitive samples are required.

It was only in 1960 (156) that the cytological technique which is most widely used today in animal chromosome studies became available. This technique utilizes the small lymphocytes, the white cells originating in the lymph system, that are present in the circulating blood. These cells, 8-10μ in diameter and having a large nucleus, are the smallest of the white blood cells. They are involved in the carrying and production of antibodies for combating infections and are also involved in fibrous formation for healing or enclosing clots. They account for 25-30 percent of the white cells in human blood, and, for comparison, 80-90 percent of rat blood white cells.

Up until recently, lymphocytes had been thought to have lifetimes on the order of 4 hr. These estimates were based on the fact that the number of lymphocytes entering the blood system through the lymph ducts is several times greater that the total number present at any moment (157). But the number of lymphocytes leaving the blood system and passing through tissue fluids to the lymph system was underestimated. In fact, lymphocytes are continuously recirculating between the blood and the lymph. Lifetimes of 100-200 days and longer have been reported (155, 158).

Lymphocytes were long-considered as mature non-dividing cells. Since cells undergoing mitosis are required for chromosomes to be visible, this would imply that lymphocytes should definitely be eliminated from consideration as test cells. But it has been found that a small fraction of lymphocytes in circulating (peripheral) blood are engaged in DNA production at a low rate (159). Additionally, and more importantly, when lymphocytes are exposed to certain drugs in vivo or in vitro, a transformation takes place, and mature cells grow, taking on the characteristics of lymphoblasts, which are large immature slightly differentiated precursors of lymphocytes (160, 161). These transformed cells then undergo chromosome duplication and enter into mitosis (159-162). Therefore, they are suitable for chromosome preparations. This sequence of events is referred to as "lymphocyte stimulation" and the drugs as "mitogens." This action is believed to be related to the antigen-antibody action of lymphocytes.

The lymphocytes are most often exposed to the mitogenic agent after a blood sample has been withdrawn from the animal and the cells put into a suitable culture media. This means that when the animal is subjected to agents that can produce chromosome breakage, the lymphocytes are nearly all at about the same stage of the cell cycle, the pre-DNA-synthesis resting stage (they are mature cells and would normally, except for stimulation, not engage in any more division). This fact greatly simplifies aberration analysis of the chromosome preparations: First, variations in sensitivity of the cell to the agent with stage in the cell cycle are absent, so all cells should be equally sensitive; second, any aberrations that result from the agent will be of the chromosome type (i.e., will involve both strands of the chromosome) since duplication occurs after breakage takes place; this greatly reduces the number of aberration types that have to be considered by eliminating all those of the chromatid type (i.e., that involve only one strand of the duplicated chromosome).

These are two distinct advantages that chromosome preparations from lymphocytes have over preparations of cells from other tissues. Other advantages of using lymphocytes are the relative ease of obtaining the cells (just draw some blood), the option for doing mutiple sampling over a period of time if this is required, and the availability of information on chromosome aberration production by ionizing radiation in the lymphocytes of one animal, man, both *in vitro* and *in vivo* (155, 163-167). Additionally, chromosome aberrations produced in circulating blood lymphocytes should be indicative, in a qualitative sense, of similar damage to other cells throughout the entire body, both for ionizing radiation and for ozone. This follows from demonstrated body-wide effects of ozone and radiation exposure and the fact that production of chromosome aberrations by these agents appears to be an action that is general to all cell types.

Objectives of the Investigation

The basic purpose of this study was to provide experimental evidence that could be used to help decide whether presently permissible human exposure levels for ionizing radiation and for respirable ozone should be reduced when exposure to both agents takes place, particularly when the exposures are simultaneous. Such evidence should consist of quantitative information on the production of a specific type of biological damage in small mammals through exposure to each of the agents alone and to both of them together.

The production of chromosome aberrations in circulating blood lymphocytes was chosen as the biological indicator of damage. This choice was made on the basis of known response to radiation (in man) and probable response to ozone suggested from related information. Chromosome aberrations in these cells would be indicative of similar and related damage with long-term consequences in other cells of the body as well.

As a result of this choice, the specific objectives of this investigation (all referring to the peripheral blood lymphocytes of a particular species of small mammal) were the following:

1. determine the frequency of chromosome aberrations produced by exposure to ionizing radiation; compare this value with those available for other cells in the same species and for lymphocytes in the other species;
2. determine whether chromosome aberrations are produced when the animals undergo exposure to ozone at a concentration approximating that presently permitted for human exposure and for a duration that related information suggests is adequate to produce sizeable numbers of aberrations; if aberrations are seen, determine the frequency of production by ozone;
3. determine the frequency of chromosome aberrations produced when the animals are exposed to ozone and ionizing radiation simultaneously; compare this value with that which would be expected based on exposure to each of the agents individually in order to determine whether synergism, additivity, or antagonism occurs with combined exposures;
4. attempt to assess the value of the information obtained by the experimental study in relation to the overall problem of deciding on appropriate permissible exposure levels for ozone and ionizing radiation when exposure to both agents takes place, especially simultaneously.

CHAPTER II

METHODS, MATERIALS, AND EQUIPMENT

Chinese Hamsters As the Test Animals

The species, of all the small mammals available for laboratory studies, which seemed most suitable for this investigation was the Chinese hamster, Cricetulus griseus. This choice was made primarily on the basis of the low diploid chromosome number of this species, 22 per somatic cell. See Figure 1 (168). Most other small mammals have many more; the Syrian or golden hamster, for example, has 44. Small mammals known to have fewer chromosomes such as the Tasmanian rat kangaroo with 12 or 13 (168) or the mole-vole with 17 (168) are not easily obtainable. As somatic cells from placental mammals all appear to contain approximately the same amount of genetic material (169), fewer chromosomes per cell also means larger chromosomes, both of which should facilitate analysis.

Another reason for choosing the Chinese hamster was the availability of ample numbers of laboratory-grade animals. Most U. S. commercial suppliers obtained their parent stocks from Children's Cancer Research Foundation, Boston, which has a well-established colony dating from 1952. Inbreeding has been accomplished there by brother-sister matings over many generations (170). The supplier[1] of the animals for

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[1] Chick Line Company, Vineland, New Jersey.

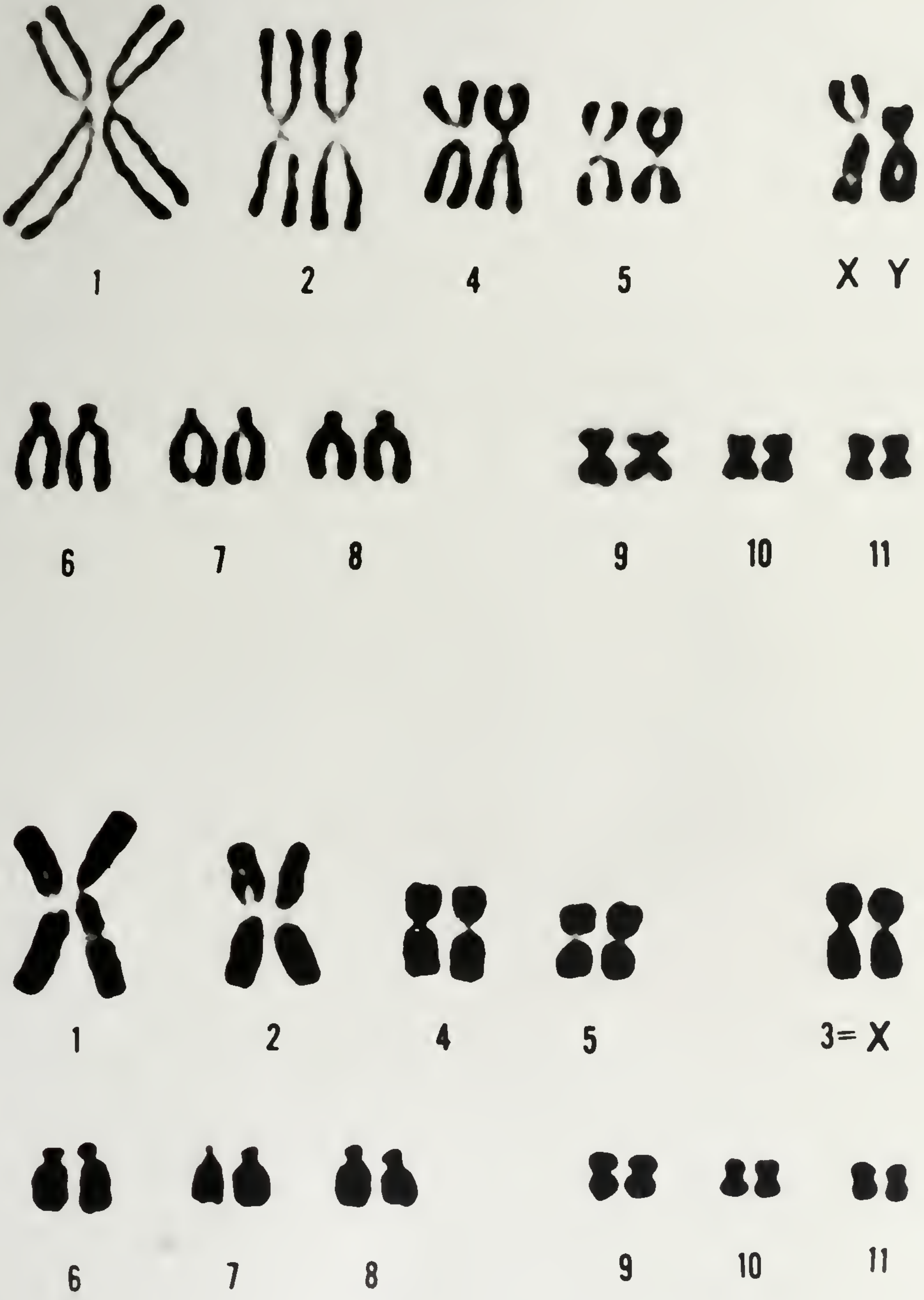

FIGURE 1. CHINESE HAMSTER CHROMOSOMES

this investigation has maintained a closed colony since 1964 (171). A third reason for the choice of this species was the availability of information on spontaneous and *in vivo* x-ray induced chromosome aberrations in bone marrow cells (172).

The Chinese hamster is smaller than the more common, golden hamster. See Figure 2 (173). Approximately 10 cm long with a 1-2 cm tail, it is gray-brown in color with a black dorsal stripe and white underside. Maturity is reached at 8-10 weeks, and animals can live at least 3-6 years (171, 173). Its outstanding characteristic is its fearlessness. When being picked up, an animal will usually not flee but may roll over and squeal angrily; handling must be gentle and without nervousness or the animal will become vicious and bite (173, 174).

The animals in this investigation were all females and had an average weight of approximately 27 g. They were obtained at an age of 8-10 weeks and were utilized at ages ranging from 13-15 weeks to 19-21 weeks for the preliminary experiments and from 11-13 weeks to 15-17 weeks for the final experiments. As required by their pugnacious nature toward one another (173), the animals were housed individually in standard rat cages and provided with paper towels for nest building. They were maintained on standard rat chow in the cage, supplemented with occasional fruit or greens, plus water *ad libitum*. Lighting was natural. No evidence of disease or parasites was seen for the 57 animals ultimately maintained over the course of the investigation. Each was earpunched for ease in identification.

The Orbital Bleeding Technique for Blood Withdrawal

Experimental design required mutiple sampling, a minimum of two blood samples from each animal. (A pre-treatment culture and at least

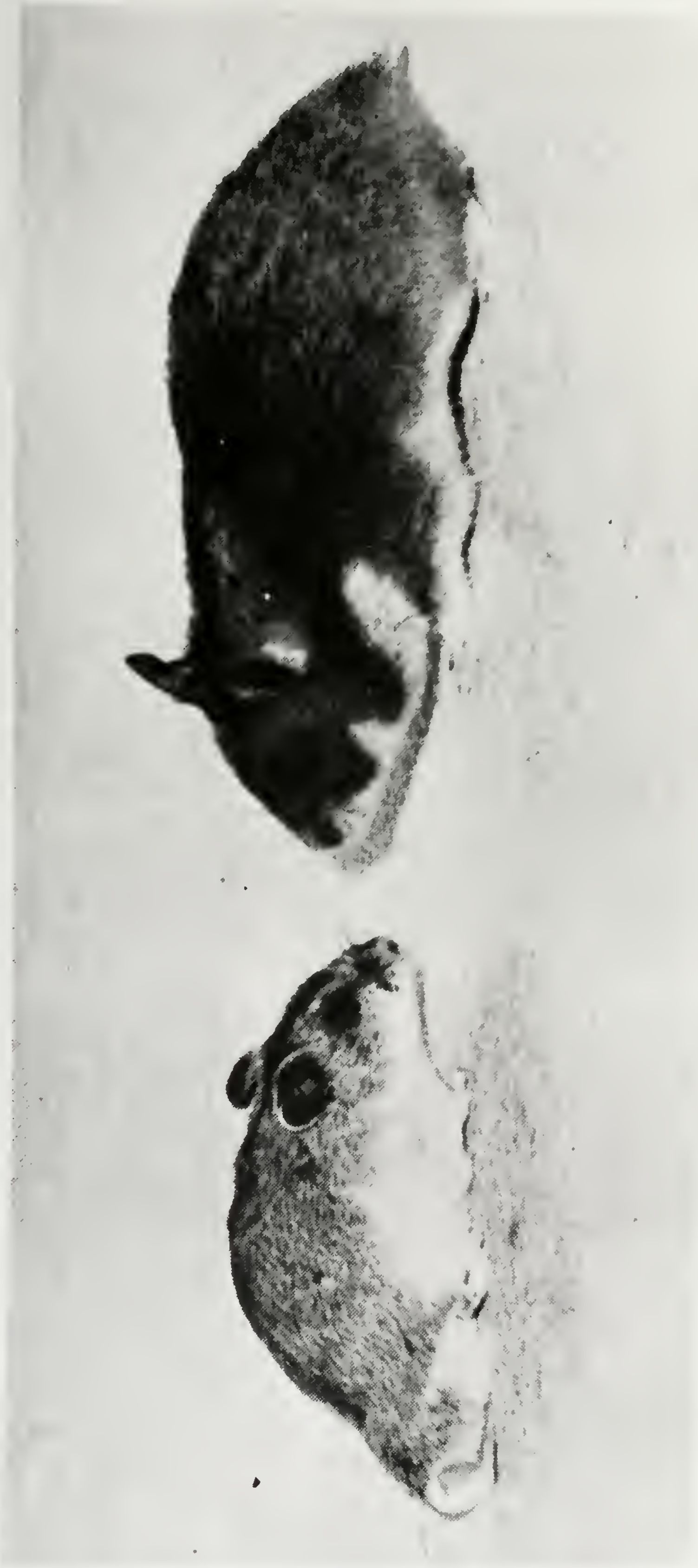

FIGURE 2. CHINESE AND GOLDEN HAMSTERS-COMPARATIVE SIZE

one post-treatment culture were required.) Because of this, traumatic techniques were to be avoided. The small size of the animal dictated that the blood samples would likewise have to be small, on the order of 0.2 ml, which, based on mouse data, could represent approximately 10 percent of the total blood volume (175, 176). Additional requirements, imposed by the culturing to follow, were that the technique not result in coagulation, hemolysis, or contamination of the blood.

These requirements, along with hamster body structure and a desire for simplicity, eliminated the more standard techniques for small animals such as tail bleeding, toe removal, and cardiac or jugular vein puncture (175). The technique which seemed most satisfactory and which was employed for the investigation was the orbital bleeding method (175, 176). This technique is based on the fact that a venous plexus lines the rear of each eye orbit. The capillaries forming this network are fragile and are easily ruptured. Slight pressure from a thin blood collecting tube with polished tip[2] results in hemorrhage. Blood accumulates in the orbit, which serves as a reservoir, and the blood tube fills through capillary action after being withdrawn slightly. See Figure 3 (176). Blood flow stops when the tube is withdrawn and the eyeball reestablishes normal ocular pressure on the venous network.

The technique is demonstrated in Figure 4 (176). The loose skin of the head is drawn tight to make the eye bulge, taking care not to stop respiration, and the pipette tip is inserted in the inner corner. It is slid in and back along a bony shelf until the plexus is reached.

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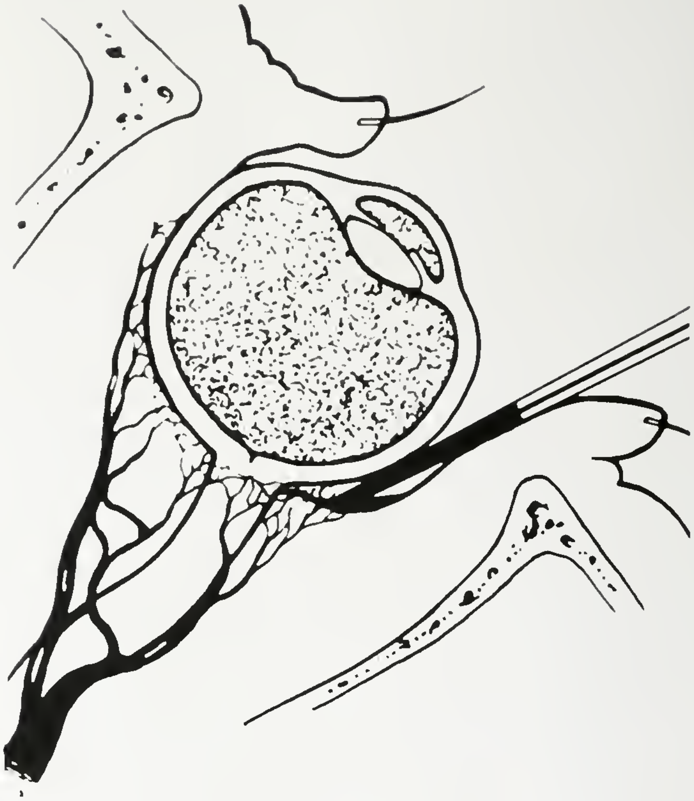
[2] Natelson Blood Collecting Pipettes, Non-heparinized, Scientific Products Division of American Hospital Supply Corporation, Evanston, Illinois.

FIGURE 3.

DIAGRAM OF BLOOD PIPETTE IN EYE ORBIT

FIGURE 4.

ANIMAL POSITIONING AND PIPETTE INSERTION FOR ORBITAL BLEEDING TECHNIQUE



The tube, 3 mm OD, 1.5 mm ID x 150 mm, fills in a few seconds when a twisting motion is used. Holding approximately 0.26 ml when filled, it is held horizontally and then tipped to a vertical position for emptying. The tube interior was moistened with heparin solution (See Appendix 1) immediately before use; the culturing to follow precluded the use of pre-coated tubes, which included preservative in the heparin.

Bleeding could be done from either eye. Neither vision nor health appeared to be damaged in any way as a result of this non-traumatic technique which could be performed repeatedly on an animal, even on successive days. This agrees with what has been observed for mice (176).

The Culture of Peripheral Blood Lymphocytes

One of the main difficulties encountered in this investigation was the development of a technique for the culture of lymphocytes from the small inoculums of Chinese hamster blood. Principally, this meant determining what modifications of existing techniques were required in order to produce successful cultures in this laboratory, for lymphocyte culturing must still be considered as an art. A brief summary of the technical developments which led to the procedure utilized in this study is presented below in order to point out some of the difficulties involved.

The first successful stimulation of lymphocytes from peripheral blood in culture media was reported in 1960 (156, 177). The technique used 10 ml inoculums of human blood and required removal of erythrocytes before culturing. The mitogen employed was phytohemagglutinin (PHA), an extract of ordinary beans. Subsequent experimentation with animals indicated that while the method, using somewhat smaller inoculums for small animals, could be successfully employed for some, considerable modification was required for success with others, and some defied all attempts at modification (160). Particular difficulty was experienced with mice, rats, and hamsters.

Partial success with golden hamsters was reported in 1963 with a procedure that involved removal of the leucocytes from whole blood, leaving erythrocytes plus plasma, and the culturing of the leucocytes in a media which employed calf serum as a replacement for the hamster plasma (178). In 1965, using rats, it was discovered that a more complete separation of the leucocytes from the plasma, done by serial washings with normal saline, resulted in greater mitotic index in cultures (179). But even this did not bring about consistently successful mouse and hamster cultures.

Concurrent with these attempts, work was also being done to develop culturing techniques which would employ very small blood samples, which would not require leucocyte separation from whole blood (with the loss of mitotically competent cells - a frequent occurrence), and which would be less subject to unpredictable failures. A method was reported by Hungerford in 1965 that accomplished all these points (180). It utilized a specially formulated culture media (See Appendix 1) and an improved hypotonic treatment for swelling the mitotic cells. It was developed for human blood and was also used for birds.

Finally, in 1968, complete plasma removal (through high dilution and centrifugation) was combined with Hungerford's innovative method to yield a microtechnique that was reported by Buckton and Nettesheim to by consistently successful for mouse blood (181). Through inquiry, it was learned that the same technique had been successfully applied to

Chinese hamsters (182). Consequently, the method of Buckton and Nettesheim was chosen to serve as a starting point in developing a method to yield successful cultures in this laboratory. The main modifications instituted were to substitute orbital bleeding for cardiac puncture, to halve the amount of whole blood used (0.15-0.25 ml instead of 0.30-0.50 ml), and to extend the incubation time by 50 percent (3 days instead of 2 days). The complete procedure is presented in Chapter III.

Recently, a preliminary report of an additional method for culturing peripheral blood lymphocytes from Chinese hamsters was issued (183). It differs most from the method used in this investigation by its use of extremely small quantities, 1-2 drops, of unseparated whole blood.

The Production and Quantization of the Ozone Environment

The principal methods of ozone production are electrochemical dissociation, corona discharge, silent electric discharge, and short-wave ultraviolet irradiation of oxygen. From these, ultraviolet irradiation (UV) was chosen for this investigation. This was to avoid simultaneous production of oxides of nitrogen (129), for these can influence biological response (91).

Both production and decomposition of ozone occur when oxygen is irradiated with ultraviolet light (129):

Production

$$O_2 + h\nu \rightarrow 2\ O \quad \ldots\ldots\ldots\ldots\ldots\ldots (14)$$

followed by

$$O_2 + O \rightarrow O_3 \quad \ldots\ldots\ldots\ldots\ldots\ldots (15)$$

or

$$3\ O \rightarrow O_3 \quad \ldots\ldots\ldots\ldots\ldots\ldots (16)$$

Decomposition

$$2\ O_3 + h\nu \longrightarrow 2\ O_2 + 2\ O \longrightarrow 3\ O_2 \qquad \text{...........(17)}$$

For 14, wavelengths <2450 Å are required to provide sufficient energy while 17 occurs with a 2537 Å wavelength where ozone absorbs strongly.

A mercury vapor discharge produces its strongest (resonance) radiations at 1849 Å and 2537 Å. It is a suitable UV source for ozone production when it is enclosed in quartz rather than glass so that the shorter wavelengths will be transmitted. Such a bulb[3] was utilized.

Ozone production took place within the chamber used for animal exposures and irradiations. Figure 5 is an overall view of the experimental setup including exposure chamber, x-ray source, ozone detector, and related equipment. The ozone bulb was placed at the rear and on the central axis of the 46 cm ID x 92 cm chamber. See Figure 6. The animals, in a ventilated cage near the center of the chamber, were shielded from the UV light by a 37 cm diameter aluminum sheet. See Figure 7. Air which flowed continuously from the rear of the chamber to its front at a rate of 1.5 l./min served to transport ozone to the animal cage.

Ozone concentration was varied by altering the voltage impressed across the UV bulb and its series ballast (a 40 W light bulb) by use of an autotransformer. Response was linear. Unwanted variation was avoided by operating the autotransformer from the output of a constant voltage transformer.

The ozone concentration of interest within the exposure chamber was that in the animal cage. This was determined continuously through

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[3] No. G4S11, General Electric Company, Cleveland, Ohio.

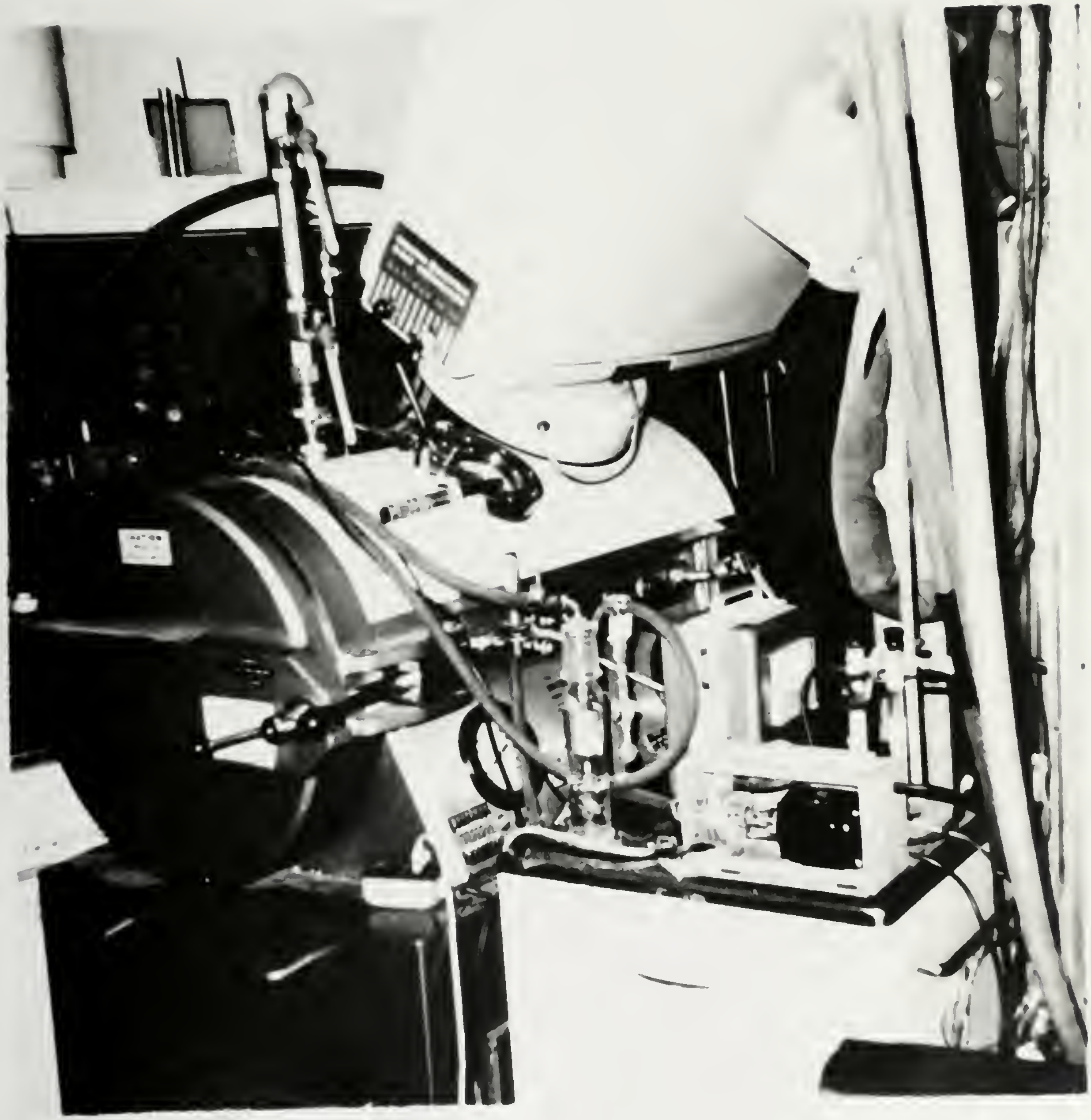

FIGURE 5. OVERALL VIEW OF ANIMAL EXPOSURE SETUP

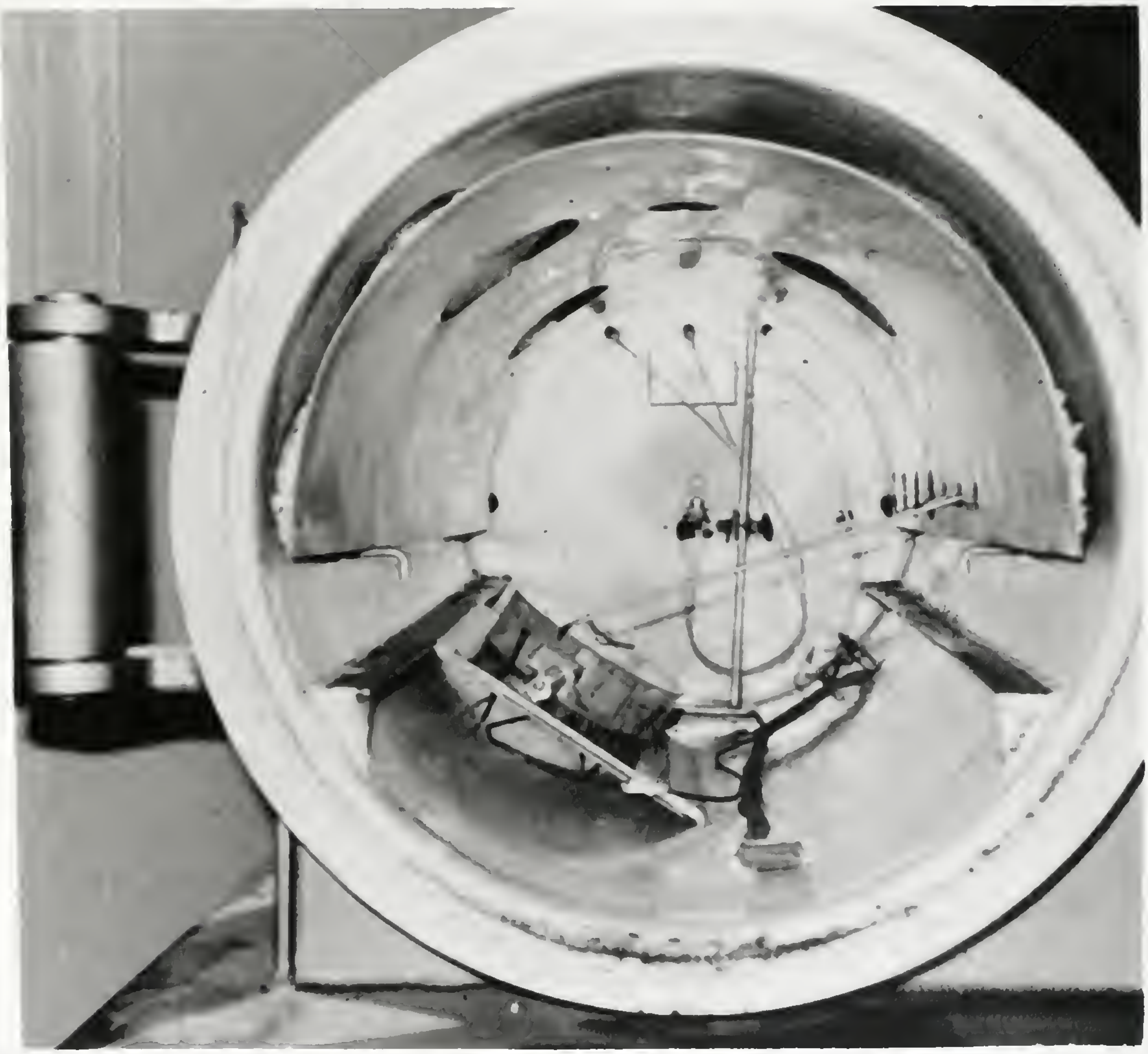

FIGURE 6. THE INTERIOR OF THE EXPOSURE CHAMBER WITHOUT UV SHIELD

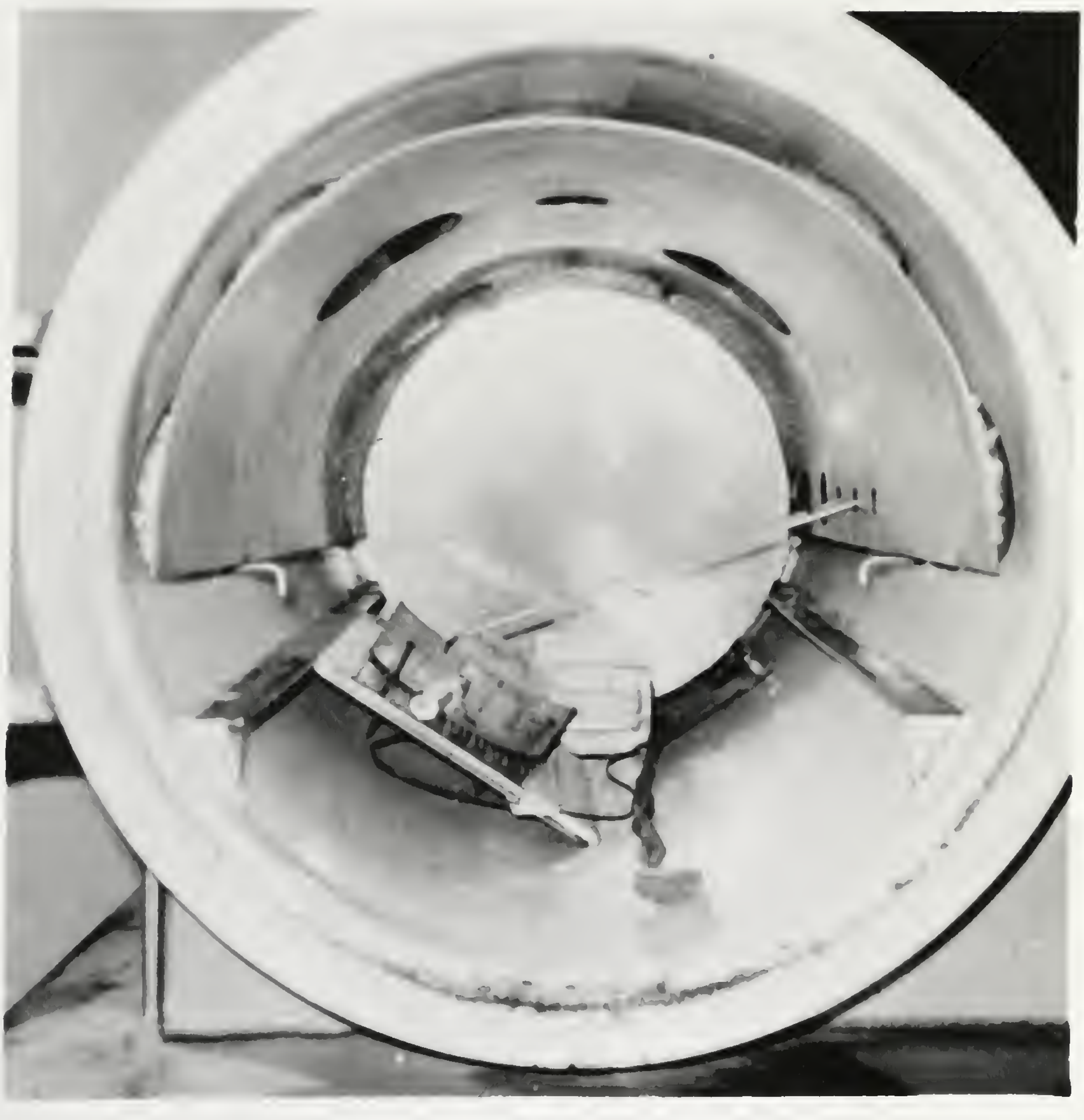

FIGURE 7. THE INTERIOR OF THE EXPOSURE CHAMBER WITH UV SHIELD

the use of a Mast Ozone Meter[4] connected, through a valve in the chamber, to a 3.2 mm ID stainless steel sampling tube which terminated in an unoccupied compartment of the animal cage. See Figure 8.

The Mast Meter utilizes a microcoulomb sensor (184). The sensing solution containing potassium iodide (KI) (See Appendix 1) enters the detector at a fixed rate and flows down an electrode support in a thin film. Air enters the detector at a fixed rate and comes in intimate contact with the solution film. When ozone is present and chemical reactions occur (See below), current flows, the anode and cathode having a constant potential between them. This current is measured on a microammeter in the external circuit. The reactions involved are the following (184):

Oxidation of KI in the sensing solution

$$O_3 + 2\ KI + H_2O \longrightarrow O_2 + I_2 + 2\ KOH \text{ ....} (18)$$

Production of H_2 layer by polarization at the cathode

$$2e + 2\ H^+ \longrightarrow H_2 \text{ ....} (19)$$

Reaction of H_2 and I_2 at the cathode

$$H_2 + I_2 \longrightarrow 2HI \text{ ....} (20)$$

Equation 20 is followed by a repolarization current of two electrons flowing in the external circuit. Hence the current is directly proportional to the number of ozone molecules reacting.

Ozone concentrations measured with Mast Meters were reported to be between 65 percent and 100 percent, depending on their calibration, of values determined by the neutral KI chemical method of determination,

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[4] Model 724-2, Mast Development Company, Davenport, Iowa.

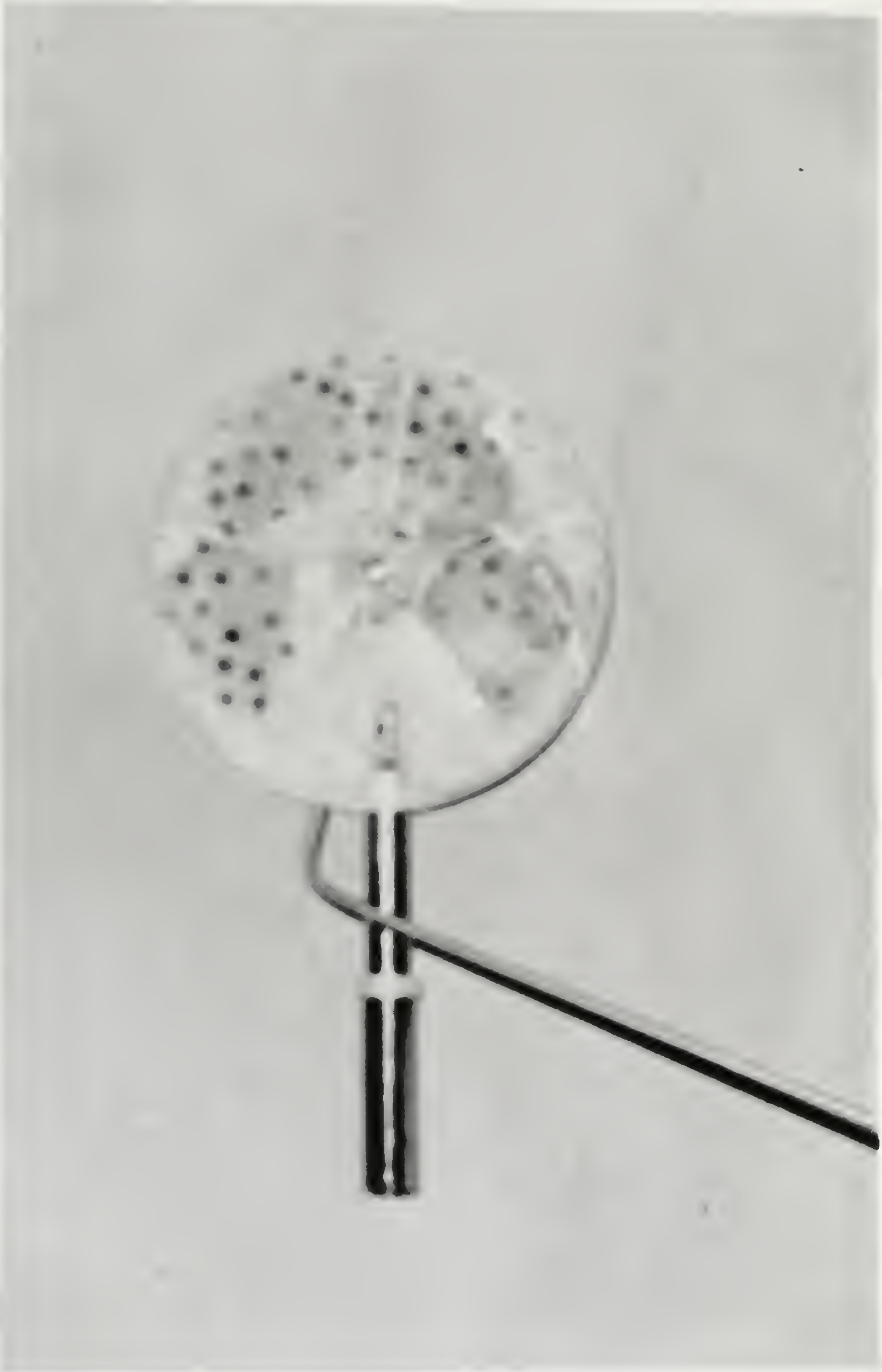


FIGURE 8. THE EXPOSURE CAGE WITH ANIMALS, R-CHAMBER, AND OZONE SAMPLING PROBE

considered as the reference method (84, 185). Consequently, it was necessary to determine the calibration of the particular Mast Meter being used. For this, another standardized procedure, the alkaline KI method, was used (185). See Appendix 2. It had some advantages over the neutral KI method, and data could be converted to standard values (neutral KI values) by multiplying by 1.54 (84).

For the calibration, ozone was produced by the UV bulb in a 2 l. beaker. The Mast Meter sampling tube and the inlet for the sampling train used in determining ozone by the alkaline KI chemical method both drew air from the beaker at the same position. See Figure 9. The bypass of the midget impinger containing the sampling reagent was used to keep the air flow from the beaker, and hence the ozone concentration in the beaker, from altering drastically when sampling began. A strip chart recorder was used with the Mast Meter so that average readings could be determined for the time periods during which samples were collected. The results of the calibration procedure are shown in Figure 10.

The autotransformer and ballast bulb for ozone production regulation, the strip chart recorder for the Mast Meter, and the compressed air tank and flowmeter for air flow through the chamber were all located outside the laboratory containing the exposure chamber so that observation and regulation could be done during an x-ray exposure. An exhaust pump for the chamber was required to keep it from pressurizing slightly, which would increase the air flow into the Mast Meter and change its calibration. This pump was located in the exposure laboratory but did not require frequent adjustment. Humidity within the chamber was kept nearly constant by passing the inflowing air through a water bubbler

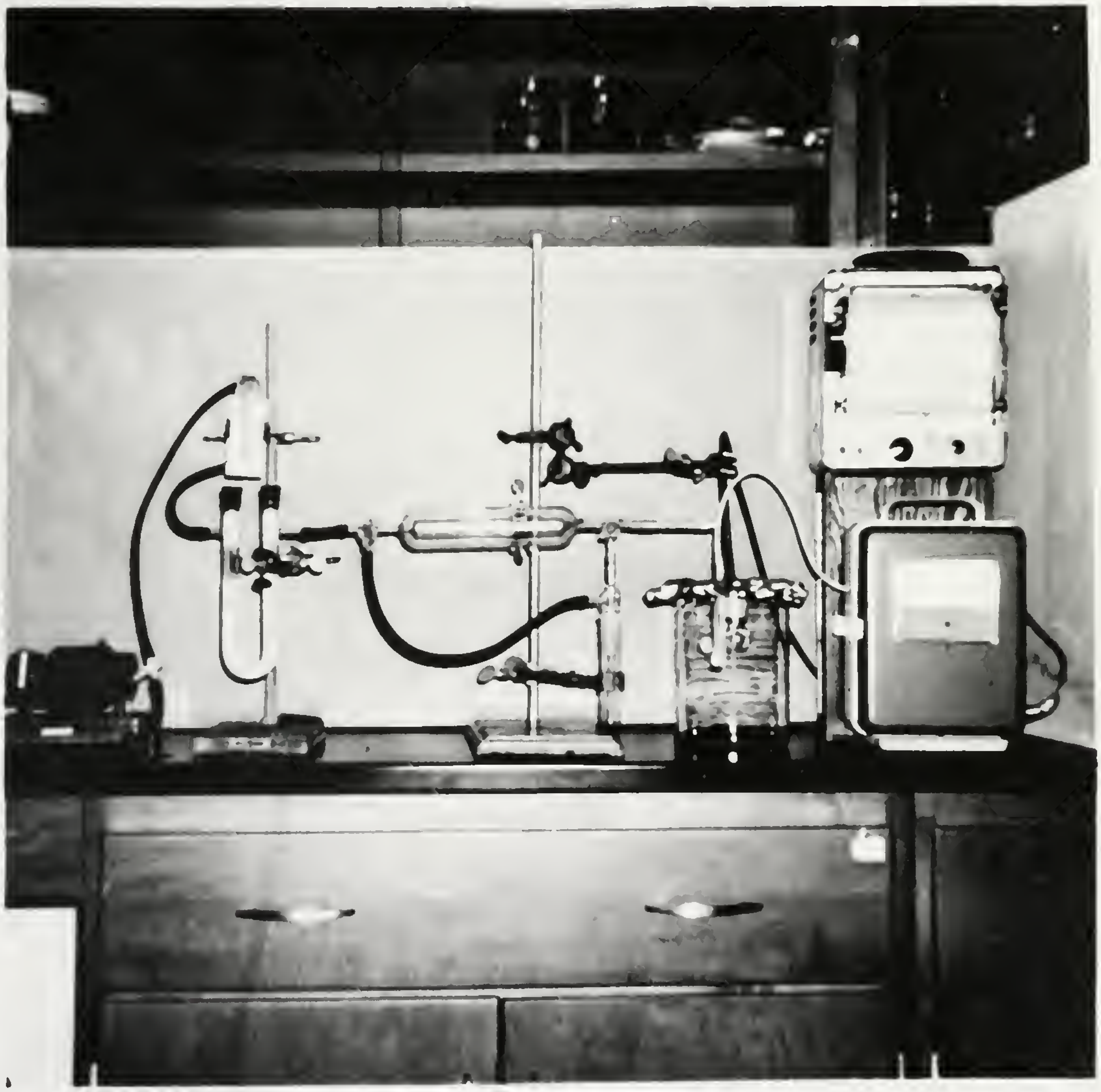
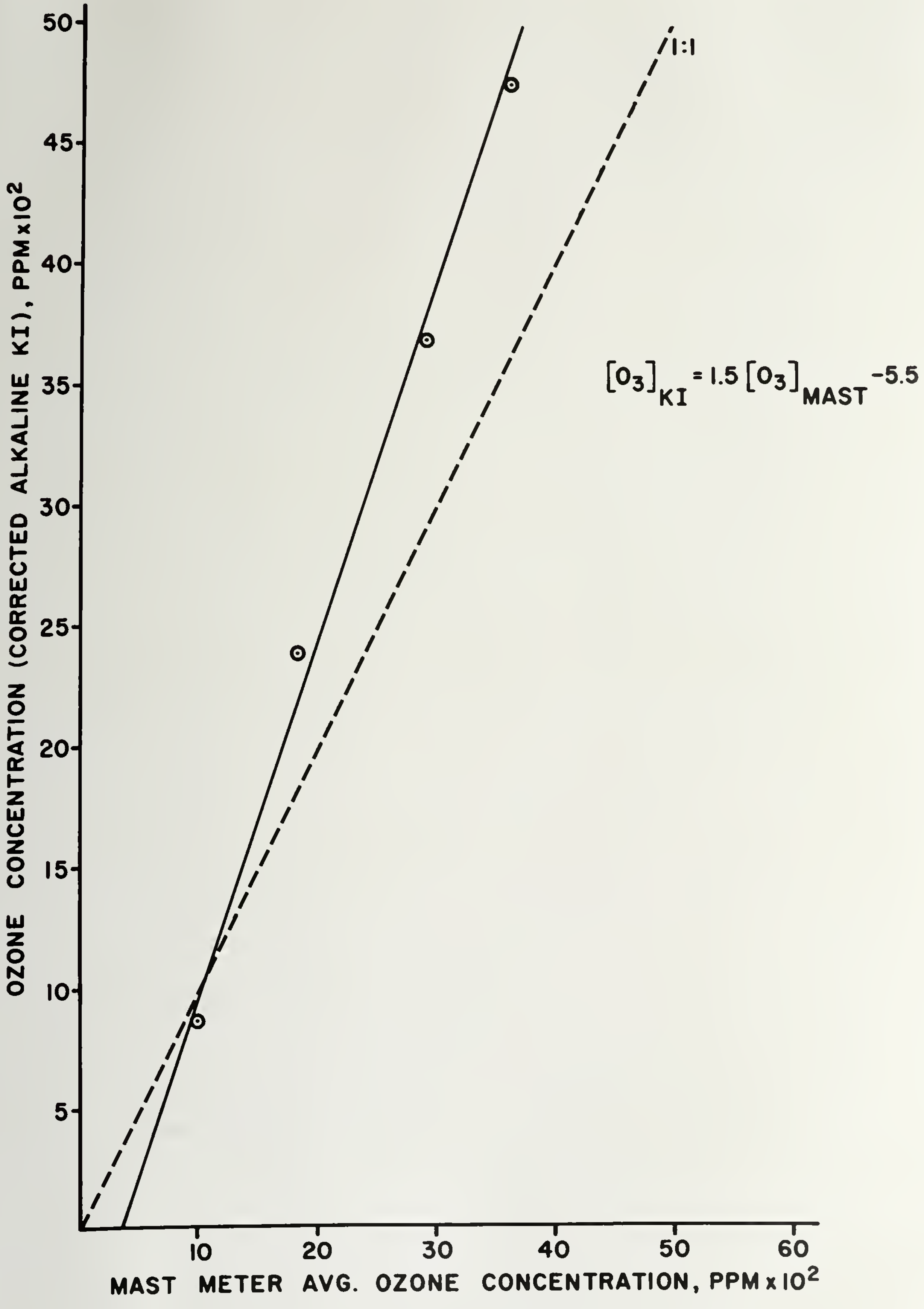


FIGURE 9. THE SETUP USED FOR CALIBRATING THE OZONE METER

FIGURE 10.

ACTUAL OZONE CONCENTRATION VS. MAST
METER INDICATED OZONE CONCENTRATION


OZONE CONCENTRATION (CORRECTED ALKALINE KI), PPM x 10^2
50
45
40
35
30
25
20
15
10
5
10
20
30
40
50
60
MAST METER AVG. OZONE CONCENTRATION, PPM x 10^2
1:1
$[O_3]_{KI} = 1.5\,[O_3]_{MAST} - 5.5$

bottle. Temperature regulation was automatic, the sensing thermocouple being in contact with the animal cage bottom at is center. Air flow into the Mast Meter was checked periodically both with and without air flow into the chamber and was found to remain constant.

Many materials react with ozone when first exposed to it or when exposed to it again after a long period of non-exposure. This reaction lowers the ozone concentration in the air. The reduction can be very large with some substances. Exposure to relatively high concentrations of ozone (up to 1 ppm) over extended periods (several hours) will eliminate the reactivity of many items. This is referred to as "ozone-conditioning" (186). The exposure chamber, the animal cage, and the sampling line were "ozone-conditioned" in order to attain the desired concentrations and to prevent low readings due to reaction in the sampling system.

The Production and Quantization of the Radiation Field

To produce the desired external radiation field, a General Electric Maximar 250-III therapy x-ray machine[5] was employed. The unit was equipped with an adjustable rectangular collimator to alter field size plus an internal light to check the field size at the object being irradiated. There was provision for inserting standard (supplied) filters into the beam plus a slot for additional filters as required. The control console was located outside of the exposure room, whose walls were lined with 1.6 mm thich lead sheeting.

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[5] Type 1, Serial No. 467617, General Electric Company, Milwaukee, Wisconsin.

Radiation was beamed into the exposure chamber through a 10 cm diameter viewing port. See Figure 5, page 35. The beam axis was perpendicular to the central plane of the 15 cm diameter lucite animal cage, and the target-cage distance was 70 cm. The cage permitted some animal movement but not toward or away from x-ray source. To monitor the consistency of the beam during an exposure period, a Victoreen[6] Rate Meter, Model 510, was employed in conjunction with a strip chart recorder. The probe, Model 601 (30-400 keV effective), was placed outside of the exposure chamber, at the edge of the round viewing port, and in the corner of the beam (which measured 11 cm x 11 cm there). To determine the total exposure during a period, a Victoreen R-chamber, Model 154 (250 R, No. x915), and Model 570 reader were utilized. The chamber was placed in the unused compartment of the animal cage. See Figure 8, page 39. A correction factor of 0.98 was applied to all readings of the chamber. This was a sensitivity factor determined by comparison of the chamber with one calibrated by the National Bureau of Standards.

The uniformity of the field over the cage area was checked in two ways, with sheet film and with an array of thermoluminescent (TLD) powder dosimeters. The film, Kodak Type AA Industrial in a leaded cassette, was placed at the cage position perpendicular to the x-ray beam. The circular rather than oblong pattern showed the beam definitely was perpendicular to the cage; densitometric measurements revealed the 90 percent isodose curve to be very nearly a perfect circle with an area larger than that of the circular cage. There were two 3.1 mm OD x 2.5 cm

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[6] Victoreen Instrument Company, Cleveland, Ohio.

TLD dosimeters per wedge-shaped animal compartment (with two readings per dosimeter). They were positioned in the midplane of the cage in two concentric circles (7 cm and 14 cm diameters) and surrounded with rice as phantom material. Response was linear with dose in the region of interest, 9-17 R. The average dose was determined for each animal compartment; the maximum deviation of animal dose from the mean dose was 6 percent of the mean.

The x-ray machine was operated with 200 AC volts across the primary of the high voltage transformer, this corresponding to approximately 200 KVP. The standard filter employed was 2 mm Cu plus 2 mm Al. With this combination of voltage and filtration, the half-value layer (HVL) was determined to be 2.35 mm Cu, equivalent to an effective beam energy of approximately 118 keV. This ensured a maximum decrease in dose of approximately 15 percent over a 4 cm animal depth with the 70 cm target-to-cage distance used (187). The unit was operated at 5-9 mA with 2 mm Cu additional filtration which served to reduce the intensity of the beam down to the desired level, approximately 1 R/min at the animal position. Current was adjusted as necessary during an exposure to keep the exposure rate, seen on the ratemeter and strip chart, at the desired value.

Scoring Chromosome Aberrations

The aberrations of interest in this investigation are all of the chromosome type. They involve both strands of the duplicated chromosomes, since the breaking and rejoining associated with exposure of the animals to ozone and/or radiation occurred before duplication of the lymphocyte chromosomes.

The various chromosome-type aberrations are shown in Figure 11 (93). The following discussion refers to this figure.

The chromosome aberrations of interest in a quantitative investigation are those that require a minimum of subjective judgement on the part of the observer in order to identify them. Consequently, types which involve changes in the lengths of the arms (the strand portions on each side of the constriction, or centromere) should usually not be included in scoring (counting). These include columns B, C, and F. Each of these types involves two breaks and an exchange of resulting arm segments to give chromosomes that in most cases give an outward appearance of being normal. Columns B and C, involving one chromosome, are referred to as inversions, and these were never scored (counted). Column F, involving two separate chromosomes, is referred to as a translocation. This was scored (as two breaks) only if the arms of one of the chromosomes were decidedly and distinctly much longer than they should have been. See Figure 1, page 25. Arms shorter than normal would not necessarily indicate a translocation, but could result from a simple chromosome break, as shown in Column A.

The types of aberrations which were the basis of scoring are shown in Columns A, D, E, and F. They are all easily identified.

Column A, called a simple break or a deletion, results from one chromosome being broken and does not require any type of recombination in order to appear. It was scored as one break on the basis of finding acentric fragments (or one fragment as shown in A4), fragments without a centromere. Deletions were the aberration type seen most frequently.

Columns D, E, and F each involve two breaks and were so scored when found. Columns D and E, each involving a deletion from a single

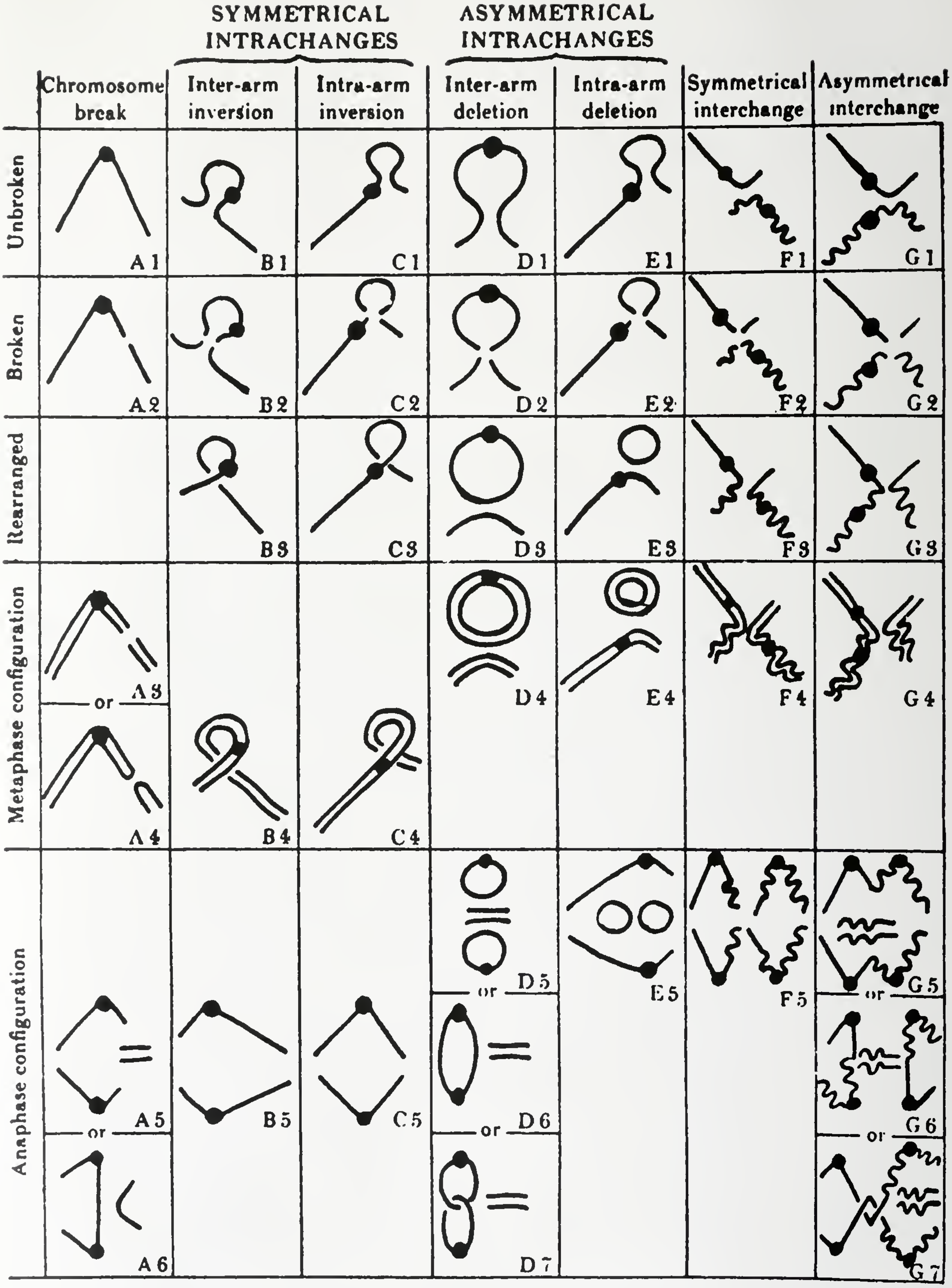

FIGURE 11. DIAGRAM OF THE VARIOUS CHROMOSOME-TYPE CHROMOSOME ABERRATIONS

chromosome, result in the formation of ring structures, either with or without a centromere; these aberrations are referred to as rings and were scored on the basis of finding the rings. Column F, involving an interchange between two chromosomes, results in the formation of a chromosome with two centromeres; it is called a dicentric and was scored on the basis of finding dicentric chromosomes.

In summary, a deletion was scored as one break on the basis of finding accentric fragments, a gross translocation was scored as two breaks on the basis of finding a chromosome with exceptionally long arms, while rings and dicentrics were scored as two breaks each when found. Figures involving three breaks or more are possible but were not seen.

Estimating the Ozone and Radiation Exposures Required

The principal objective in choosing the ozone and radiation exposure levels was to have exposure to one agent alone or to the two simultaneously result in frequencies of chromosome aberration that were significantly different statistically.

The production of chromosome aberrations by sparcely ionizing radiation such as x-ray is a random process. With a given dose, the frequencies of cells in the "one-break," "two-break," etc. classifications follow the Poisson distribution (155). A given ozone exposure would likewise be expected to result in a Poisson distribution of chromosome breaks per cell since ozone, like x-ray, is believed to act primarily through oxidation events associated with free radical production (154).

The number of breaks per unit of cells (such as 100 cells) should also follow the Poisson distribution. The number of breaks, n, is a point estimate of the average number of breaks, u. If $u > 15$, the Poisson can be approximated by a normal distribution with a mean = u and standard deviation = $u^{\frac{1}{2}}$. The sample (estimated) mean would be n and the sample standard deviation would be $n^{\frac{1}{2}}$. On a breaks per cell basis, the sample mean would be n/N, the sample standard deviation $n^{\frac{1}{2}}/N$, and the 95 percent confidence interval $n/N \pm 2\, n^{\frac{1}{2}}/N$, where N = the number of cells in the unit. The statistical significance of the difference between two means could be determined through the use of Student's T distribution or by determining whether the confidence intervals around the means overlapped, a stricter test.

The ozone and radiation exposure levels were chosen to yield equal break frequencies, with the total number of breaks for each being large enough for the normal distribution approximation to apply. The levels were also chosen so that break frequency for combined ozone plus radiation exposure, expected to be approximately twice that for either agent alone, would be significantly different statistically from the frequency for either agent alone, as determined by the confidence interval overlap method. The levels were also chosen to be as low as possible while still meeting these requirements.

The initial estimates of chromosome break frequencies from x-ray exposure were made on the basis of available information on the response of human lymphocytes to x-radiation _in vivo_ and _in vitro_ (155, 165). Supporting evidence came from chromatid aberration data for the _in vivo_ irradiation of Chinese hamster bone marrow cells (172), taking into account that the cells examined were in the post-DNA-replication stage of the cell cycle at the time of the irradiation and hence had greater radiosensitivity (disregarding differences in cell type) (148).

For ozone, deletion frequency was estimated on the basis of the exposure of human cell cultures to high concentrations of ozone by Fetner (154), while ring and dicentric frequency was strictly a guess based on analogy with radiation. Fetner's ozone data were on chromatid deletions. In arriving at the estimated frequency for this investigation, the pre-DNA-synthesis stage of the cell cycle (G_1) was assumed to be approximately one-half as sensitive as the post-DNA-synthesis (G_2) stage (148). (Difference in cell type was disregarded.) Additional assumptions included linear dependence of break frequency on exposure time, to be conservative (the data showed exponential dependence), and validity of concentration x time = constant response (84) from the 8 ppm used down to 0.1 ppm, the approximate concentration desired.

The following were the initial estimates:

Radiation

$$1 \times 10^{-3} \, \frac{\text{deletions}}{\text{cell-rad}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (21)$$

$$6 \times 10^{-6} \, \frac{\text{dicentrics + rings}}{\text{cell-rad}^2} \quad \ldots\ldots\ldots\ldots\ldots (22)$$

Ozone

$$3 \times 10^{-3} \, \frac{\text{deletions}}{\text{cell-(ppm-min)}} \quad \ldots\ldots\ldots\ldots\ldots (23)$$

$$7 \times 10^{-6} \, \frac{\text{dicentrics + rings}}{\text{cell-(ppm-min)}^2} \quad \ldots\ldots\ldots\ldots\ldots (24)$$

The dependence of dicentric or ring frequency on the square of the dose follows from two breaks being required for each, the probability for each break being linearly dependent on dose, and the occurrence of each being independent of the other. Data on spontaneous aberrations in Chinese hamster bone marrow (172) and in human blood lymphocytes (163, 166) indicated the frequency was so low ($\lesssim 1 \times 10^{-3}$ breaks/cell) that it could be neglected, but pre-exposure cultures were made anyway as a check.

The data of Hungerford (180) for human blood and Buckton and Nettesheim (181) for mouse blood indicated that it would not be unreasonable to expect, per culture, 200 metaphase spreads that would be suitable for analysis. Assuming 200 cells per culture, it was determined by trial and error using the coefficients in 21-24 and the expression for 95 percent confidence interval that 5 hr at 0.1 ppm ozone and 100 rad (over 5 hr) would be suitable exposures. These levels would result in confidence intervals of 0.22 ± 0.07 breaks/cell for ozone or radiation alone and 0.44 ± 0.09 breaks/cell for ozone plus radiation (assuming additivity) and these intervals do not overlap. Halving each exposure (to 2.5 hr at 0.1 ppm and 50 rad) would result in confidence intervals that would overlap, so the higher values, 0.5 ppm-hr and 100 rad, were the ones of choice.

To determine the suitability of these values, preliminary experiments were conducted utilizing one or two animals at a time exposed either to ozone or to radiation. The ozone exposure levels were approximately 0.45 and 0.50 ppm-hr and the radiation doses were 85, 153, and 166 rad (Roentgen-to-rad conversion followed NBS Handbook 85, Physical Aspects of Irradiation). Eight animals were involved.

Discounting the 13 and 16 day delays between irradiation and successful cultures for animals receiving 153 and 166 rad, these delays in part a result of culturing oven temperature variations, the deletion yield for irradiation was only 65 percent of the expected yield. Similarly, for the ozone-exposed animals, the deletion yield was only 62 percent of the expected value. Additionally, it was found that the average number of metaphase spreads per slide that were suitable for analysis was nearer to 40 than to 200, with values ranging from 10-71.

Since the basic exposure group was to have four animals, there was a possibility that fewer than 15 deletions would be found for all the animals pooled. Even if the exposures were raised to correct for the lower-than-expected yields, only 10 deletions per 100 cells would be expected, so the average of 160 spreads per animal group would only give an average of 16 deletions. Fewer than 15 would mean that the normal distribution approximation to the Poisson distribution could not be used, and that confidence intervals would not be appropriate. The breaks that would result from dicentrics and rings were not being counted on to raise the total breaks observed. This was because the radiation and the ozone were being administered over a relatively long time period, 5 hr, which may be longer than the average lifetime of a chromosome break (155). This would mean that breaks created at the beginning of the period might not still be available for combination at the end of the period, so the yields of multiple-break aberrations would be lowered.

Consequently, the decision was made to raise the ozone and the radiation levels to correct for the low yields and to increase the expected number of deletions from 10 up to 15 per 100 cells, for four animals would certainly be expected to yield 100 analyzable spreads. It was also decided to increase the ozone concentration rather than increase the exposure time to >5 hr.

The radiation dose to be used, then, was (100 rads)(1.5/0.65) = 231 rad and the ozone concentration would be (0.1 ppm)(1.5/0.62) = 0.24 ppm but a decision was made to limit the ozone concentration to 0.2 ppm, for a total exposure of 1.0 ppm-hr. This was done to avoid the possibility of any observable effect on the well-being of the animals, i.e., to keep the exposure level at a value known to be tolerable (84).

For each condition, ozone, radiation, and ozone plus radiation, there were two exposure groups of four animals each. An additional group was put through the entire procedure minus the agents (ozone and/or radiation) to serve as a control. Also, another group was subjected to a 335 rad radiation exposure, approximately 50 percent greater than the dose the others received. This was done to demonstrate that break frequency does in fact increase with radiation dose. A preliminary report indicated that 335 rad was one-third of the $LD_{50/30}$ for 100 day old Chinese hamsters (188), so complications which would interfere with the procedures to follow were not expected and did not appear.

CHAPTER III

PROCEDURES

This chapter presents a step-by-step description of the numerous operations that were involved in this investigation. For some of these operations, details were important, so the entire procedure is presented. Reference is also made to the appendices for various formulations and supplementary procedures. Operations are presented in their normal time sequency.

Animal Exposures

The four animals in each exposure group were randomly selected from among those for which successful pre-treatment cultures were available. Since the animal quarters were located near the exposure room, the animals remained in their boarding cages until transferred to individual compartments of the exposure cage immediately prior to beginning an exposure. Each animal occupied approximately 50-70 percent of the space of its compartment. Exposures began approximately at 9 A.M. and were of a 5 hr duration.

If the exposure involved ozone, the UV generator remained operating the previous night so the chamber would have an ozone environment ready. Similarly, if the exposure involved radiation, the ratemeter and the R-chamber reader were left operating overnight so they would be stabilized by irradiation time. The response of the ozone meter was checked with an electric discharge ozone generator outside the chamber before and after each exposure.

Air flow through the chamber was maintained at 1.5 l./min throughout each exposure. This provided a complete air change in the chamber every 100 min. Since the exposures were conducted during daylight hours, a light was provided at the second viewing port of the chamber to illuminate the interior. Temperature was maintained at 15.5°C. Relative humidity averaged 70 percent for exposures involving ozone and 45 percent for exposures to radiation alone. This difference resulted from ozone exposures being done on consecutive days and radiation exposures having at least one day between them, which gave the chamber time to air and dry out. The animals of each group were observed through the viewing port at least twice during their exposure; they were generally inactive but awake.

The chamber was opened once during each radiation exposure to read and recharge the R-chamber. This was done so that readings would be around mid-scale where they were most accurate. An air density correction factor of 0.98 was applied to the readings because the exposure chamber temperature was below 22°C. During each radiation exposure, the x-ray current was adjusted when necessary to keep the ratemeter reading at the desired level. An exposure rate of 4.8 R/min (7.0 R/min) at the ratemeter probe corresponded to the desired 0.82 R/min (1.15 R/min) at the cage R-chamber. Values in parentheses were for the high radiation group. During each ozone exposure, the UV bulb voltage was adjusted when necessary to keep the cage ozone concentration at the desired 0.2 ppm. Response was relatively rapid, concentration adjustment time varying inversely with the size of the voltage change.

Once a steady-state condition was attained, the cage ozone concentration was the same as that in the rest of the chamber. This indicated that there was no appreciable ozone degradation by the Lucite cage after it was ozone-conditioned. There was no detectable ozone production by the radiation beam, and the x-ray environment appeared to have no effect on the performance of the ozone meter.

When an exposure was completed, the animal cage was removed from the exposure chamber immediately; the animals remained in the cage awaiting the blood sampling procedure, which began promptly.

Blood Sampling

The collection of blood samples by the orbital bleeding technique was done prior to an animal being accepted for exposure (once, or more if required by culture failure or low yields of chromosome spreads), immediately after exposure, and again at intervals if required or desired. This operation and all those following were the same for pre- and post-exposure samples.

All materials and equipment contacting the blood were sterile, from the sampling operation to the cell-killing step of the slide preparation procedure. They were either purchased that way or autoclaved at 121.5°C, 15 psig for 15 min. Sterile technique was employed. Except for the tissue culture tubes, all glassware was silicone-treated to reduce cell adhesion. For cleaning glassware, the detergent employed was non-toxic to tissue cultures.[7]

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[7] 7 X Glassware Detergent, Limbro Chemical Company, New Haven, Connecticut.

Leighton-type tissue culture tubes were used. These have an 11 mm x 40 mm flat area on one side and a constriction above this area to act as a dam. For the procedure being followed, they were reported to be the only kind in which culturing was successful (181). The tubes employed were 16 mm OD x 125 mm with screw-caps. They were filled with 15 ml of heparinized TC-199 culture media (See Appendix 1) and kept on ice during the sampling procedure.

For each animal, blood was withdrawn with a heparin-wetted blood pipette and emptied into a culture tube which was then gently agitated and given a number identifying the animal and the culture from that animal. Generally, one pipette-full, approximately 0.25 ml, of blood was put into each culture tube. Occasionally, if $<$0.20 ml was transferred in, a second pipette was used in the other eye; the total blood put into culture ranged up to 0.35 ml. After each animal was sampled (one culture tube per animal) they were returned to their boarding cages and the culture tubes were transported to the culturing laboratory.

Tissue Culture Preparation and Maintenance

The purpose of the TC-199 culture media was to greatly dilute the hamster blood plasma (approximately 1:100). The culture tubes were spun at 500 *g* for 10 min in a refrigerated centrifuge (5°C) and the supernatant liquid (TC-199 plus plasma) was pipetted out. A 4.5 ml aliquot of culture media was added to each tube immediately after removal of its supernatant liquid, and the tubes were gently agitated. The media was prepared following the specifications of Hungerford (180)

(See Appendix 1). This was followed by the addition of 0.05 ml of pokeweed mitogen solution[8] per culture tube.

The tubes were then placed in an incubator at an angle of 5° to horizontal with their screw-caps slightly open. The incubator was maintained at 37°C with a 95 percent air plus 5 percent CO_2 gas flow through it that provided a complete change every 100 min. Humidity was maintained by a constant-level water pan in the base. The tubes were momentarily removed twice per day for gentle agitation.

After 67 hr of incubation, 0.4 ml of a 2 μg/ml colchicine solution was added to each tube (See Appendix 1). This drug, a spindle poison, prevented cells in the division process from proceeding past the metaphase stage, where the chromosomes were duplicated and contracted, to the anaphase stage where each chromosome would separate into two daughter chromosomes that would migrate to opposite ends of the cell just prior to its division. In this way cells in metaphase were "stored up" as cells entered the stage while few, if any, left it (155). After 5 hr exposure to colchicine, 72 hr total incubation, the cultures were removed from the incubator, and the slide preparation procedures were begun.

Slide Preparation

After gentle agitation, each culture was transferred by pipette to a graduated 15 ml centrifuge tube and spun for 4 min at 100 g. The supernatant liquid was removed by pipetting. A 5 ml aliquot of heparinized hypotonic potassium chloride (KCl) solution (See Appendix 1), pre-warmed to 37°C, was added to each culture. Each tube was gently agitated with a mechanized tube mixer during the KCl addition to suspend the cell button. The tubes were covered and placed in the incubator at 37°C for 15 min.

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[8] Grand Island Biological Company, Grand Island, New York.

The tubes were spun again and the supernatant liquid was removed, taking care to leave the thin light-colored overlayer of the button called the buffy coat. With a gentle mechanical-mixer agitation to avoid clumping, a 5 ml portion of fixative was added to each centrifuge tube. The fixative, which caused hemolysis and killed the leucocytes, was made up of three parts absolute methanol and one part glacial acetic acid. The tubes were spun as before (4 min, 100 g), the supernatant liquid was removed, and a second 5 ml aliquot of fixative was added to each tube, again with gentle mechanical agitation. They were covered and placed in a refrigerator for 15 min.

The tubes were spun and the supernatant liquid was removed. It had no color remaining from hemoglobin. Without agitation, enough fixative was added to each tube to approximately double the volume of its cell button, which ranged from 0.05-0.10 ml.

The 25 mm x 76 mm slides to be used had previously been washed, put into a staining dish to keep them separated, submerged in distilled water, and placed in the refrigerator. When the centrifuge tubes with fixative were put in the refrigerator, the beaker of slides was removed and placed on ice, and a flow of CO_2 gas that bubbled through the water was begun.

Using a Pasteur pipette (with rubber bulb) as a rod, the cell button in a centrifuge tube was gently dispersed. The 0.1-0.2 ml suspension was then drawn into the pipette. A slide was removed from the beaker with tweezers, grasped by the edges, and tilted with one edge against adsorbent paper to remove excess water. The slide was then leveled and the suspension from the pipette was transferred to it, confining the delivery to a 24 mm x 50 mm area or less. The slide was then rapidly passed through an open flame which ignited the methanol. When it burned off, the slide was again tilted, this time being tapped against adsorbent paper to remove excess moisture. It was then placed on a slide warmer at 40°C to dry thoroughly. The water film and the burning were necessary to obtain metaphase figures (spreads) with well-separated chromosomes that lay flat on the glass.

The slide-making procedure was repeated for each of the cultures being processed. When the slides were thoroughly dry, the identifying numbers were etched on them. Each one was then partially but systematically scanned with a phase contrast microscope.[9] This was done to get a relative measure of how successful the culturing and slide preparation procedures had been by counting the number of chromosome spreads observed. Few or no spreads seen on a slide indicated that the entire procedure would have to be repeated for that particular animal. This did occur in some cases and will be discussed later.

If the quality appeared adequate, the slide was stained with Giemsa blood stain[10] (See Appendix 3). The 24 mm x 50 mm area with material was covered with a glass slip fastened with Permount.[10] The stained chromosomes appeared purple.

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[9] AO Phasestar, American Optical Corporation, Buffalo, New York.

[10] Fisher Scientific Company, Springfield, New Jersey.

Recording of Data

Each slide was scanned using bright-field microscopy. Overlapping fields of view were used to reduce the likelihood of missing a spread near the edge of the field. Scanning was done at 100x total magnification with spot checks at 950x for questionable figures. The object was to locate chromosome spreads, record their location (the microscope stage was graduated), and photograph them at 950x total magnification for later analysis. So as not to bias the data, each spread that was located was photographed, without regard to its quality.

Generally, only the 24 mm x 50 mm portion of the slide covered with a glass slip was scanned. Occasionally the preliminary phase scan indicated that the material had spread beyond the intended area; these slides were scanned over their entire area.

The microscope employed was a Leitz[11] Ortholux equipped with an Orthomat automatic 35 mm camera. Since the chromosomes were stained with Giemsa, a combination of Wratten filters[12] was used to improve the visual contrast and image quality (189). With these filters, the chromosomes appeared black on a green field. Photography was black and white.

The film-developer combination chosen was Kodak Panatomic-X and Kodak D-19 developer. This panchromatic film was extremely fine-grained for sharpness of detail and high resolving power and combined moderate contrast with adequate speed (ASA 32) (190). The recommended developers were all medium-contrast materials. To improve the imaging, a high-contrast developer was selected; of those available, D-19 was chosen since it was usually used for scientific plates and instrumentation films. Through inquiry, a suitable developing time for this combination was found to be 3.5 min at 20°C (191).

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[11] E. Leitz, Inc., New York, New York.

[12] Wratten Nos. 12 (yellow) and 44 (aqua blue), Eastman Kodak Company, Rochester, New York.

Analysis of Data

Films were left in roll form for analysis. A film strip projector was used to image the films, frame-by-frame, on a screen. When enlarged in this manner, a typical chromosome spread had a diameter of approximately 25 cm. Detail was not lost because a high degree of enlargement was possible with the extremely fine grained fine used. Scoring of aberrations was facilitated by these large-size images.

To avoid the difficulties associated with being reasonably sure that different people would score the aberrations in a given spread in the same way, all the scoring was done by one person, the author. The scoring was conducted as a blind analysis to avoid any biasing of the data. Each film was identified by number only; there was no indication as to which exposure group the data had come from. The photography log book indicated that the data on a particular film came from a particular animal, but the exposure group to which the animal belonged was not listed there.

The information recorded in the scoring log book for each frame included the total number of chromosomes, the number of chromosomes in each to two general morphological classifications, "X"'s and "V"'s (See Figure 1, page 25), and the number and kinds of chromosome aberrations. Also noted were chromatid (one-arm) breaks and stain gaps (chromosome portions that did not take up stain). A group of chromosomes was counted as a spread if it had 12 or more figures, i.e., greater than one-half of the 22 chromosomes in a normal spread; sometimes chromosomes were lost from a spread during the slide preparation procedures. A normal spread had 16 "X"-shaped chromosomes and 6 "V"-shaped figures. When necessary to aid in scoring aberrations, detailed figure counting for the various sizes of "X"-shapes was also done.

Any spread that appeared blurred was later relocated on the microscope, using the photography log book to get its coordinates. This was to check whether the spread was blurred or the photograph was poor. If the spread was clear, a new photograph was taken. Similarly, any spread that exhibited a chromosome aberration was relocated on the microscope. This was to determine whether the figure scored as an aberration was an artifact on the film or on the slide or was actually chromosomal material.

At the completion of scoring, the exposure groups were revealed and the data tabulated on an animal basis and on a group basis, listing the numbers of spreads analyzed and the numbers of deletions, dicentrics, and rings found. Cross translocations were not seen. The production-frequency coefficients were then formed and comparisons were made for the various groups.

CHAPTER IV

RESULTS

Table 2 presents the exposures received by the various treatment groups. In each case except one (Group 1), the exposure and rate or concentration were near the desired values, so variation among the six principal groups was minimal. The high radiation group (Hi X) dose was 40 percent greater than that received by the other x-ray exposed groups; also it was delivered at a 40 percent higher rate.

A total of 746 chromosome spreads were examined for breaks in the final experiment. These spreads displayed a total of 87 chromosome-type chromosome breaks and 6 aberrations which could be classified as chromatid-type chromosome breaks or as stain gaps. The results of the analysis are summarized in Table 3. In this tabulation, the ozone-exposed groups were broken down into three categories, "early," "mid," and "late." These designations refer to the time interval between treatment and sampling, 0, 6.0, and 15.5 days respectively. The multiple sampling was done to have ozone data with a delay time comparable to the delays experienced with the other groups. It was discovered during the final investigation that the radiation exposure had an inhibitory effect on the lymphocytes which resulted in unsuccessful cultures (i.e., cultures with few if any mitotic chromosome spreads). This effect appeared to diminish with time after exposure, and satisfactory cultures were obtained in all cases after $\gtrsim$ 11 days had elapsed.

TABLE 2

SUMMARY OF EXPOSURES

Treatment	Group	Radiation Dose, rad	Radiation Dose Rate, rad/min	Ozone Exposure, ppm-min (ppm-hr)	Avg $[O_3]$, ppm
O_3	1	-----	-----	89.0 (1.48)	0.30
	2	-----	-----	71.7 (1.19)	0.24
O_3 + X	3	226.5	0.872	70.7 (1.18)	0.20
	4	232.5	0.775	74.2 (1.24)	0.21
X	5	235.5	0.785	---- ------	----
	6	234.0	0.780	---- ------	----
Hi X	7	326.0	1.086	---- ------	----
Control	8	-----	-----	---- ------	----

TABLE 3

SUMMARY OF DATA

Treatment	Group	Useable Chromosome Spreads	Aberrations Scored		Total Breaks	Break Frequency, breaks/cell	Time Between Treatment and Sampling, days	
			Deletions	Rings (R) & Dicentrics (D)			Average[a]	Range
O_3(late)	1a	69	6	1(R)	8	0.116		
	2a	60	7	1(R)	9	0.150		
	total	129	13	2	17	0.132	15.5	15-16
O_3(mid)	1b	13	0	0	0	0.000		
	2b	56	2	0	2	0.036		
	total	69	2	0	2	0.029	6.0	none
O_3(early)	1c	80	5	0	5	0.063		
	2c	65	4	2(R)	8	0.123		
	total	145	9	2	13	0.090	0.0	0-1
O_3 + X	3	53	6	2(R)	10	0.189		
	4	39	6	0	6	0.154		
	total	92	12	2	16	0.174	14.8	11-18
X	5	22	2	0	2	0.091		
	6	133	8	4(R) + 1(D) = 5	18	0.135		
	total	155	10	5	20	0.129	14.5	11-16
Hi X	7	102	11	3(R) + 1(D) = 4	19	0.186	14.9	12-16
Control	8	54	0	0	0	0.000	0.3	0-7

[a] Weighted by number of useable spreads obtained.

The break frequency column was included in Table 3 to permit rough comparisons. The entries, simply breaks per cell, do not reflect either differences in exposure among the groups or, for averaging, differences in numbers of useable spreads per group. Both of these refinements were incorporated in the exposure-adjusted frequencies given in Table 4. For each treatment involving two groups, a weighted average was formed when the difference between the contributing frequencies was found to be statistically insignificant (approximately normal distribution was assumed for each frequency). Two ozone plus radiation frequencies were formed, one based on the radiation exposure only so direct comparison could be made with the frequency of the radiation treatment groups and the other based on the ozone exposure only so direct comparison could be made with the frequency of the ozone treatment groups.

Radiation Exposed Animals

Comparison of the exposure-adjusted break frequencies for radiation and for high radiation treatments in Table 4 showed them to be nearly identical, within 4 percent of one another, and to have comparable sample standard deviations with values 25 percent of the means. As would be expected, the difference between the frequencies was not statistically significant. These data suggested that the number of breaks per cell varied linearly with dose in this region, 230-330 rad. More importantly, since linear dependence had been expected, the high radiation frequency provided a check on the validity of the radiation frequency.

TABLE 4

SUMMARY OF EXPOSURE-ADJUSTED BREAK FREQUENCIES

Treatment	Group	Break Frequency per Unit Exposure, f,		Sample Standard Deviation, $s_f \times 10^3$
		$\frac{\text{breaks}}{\text{cell-ppm-min}} \times 10^3$	$\frac{\text{breaks}}{\text{cell-rad}} \times 10^3$	
O_3(late)	1a	1.31	-----	0.462
	2a	2.08	-----	0.694
	average[a]	1.67	-----	0.408
O_3(mid)	1b	0.000	-----	0.000
	2b	0.500	-----	0.352
	average[a]	0.406	-----	0.284
O_3(early)	1c	0.708	-----	0.315
	2c	1.71	-----	0.605
	average[a]	1.16	-----	0.326
$O_3 + X$[b]	3a	2.67	-----	0.843
	4a	2.07	-----	0.847
	average[a]	2.42	-----	0.604
$O_3 + X$[c]	3b	----	0.833	0.264
	4b	----	0.662	0.270
	average[a]	----	0.761	0.191
X	5	----	0.386	0.274
	6	----	0.578	0.136
	average[a]	----	0.551	0.125
Hi X	7	----	0.571	0.131

[a] Weighted by number of spreads in each group.

[b] Assuming all the breaks to have resulted from the ozone exposure.

[c] Assuming all the breaks to have resulted from the radiation exposure.

Ozone Exposed Animals

The most important fact revealed by the ozone-exposed groups was that inhaled ozone did result in chromosome breaks in circulating lymphocytes. The exposure-adjusted break frequency for O_3(late) in Table 4 definitely differed from zero ($P<0.001$, i.e., <0.1 percent chance of the frequency being zero). Comparison of the frequency for O_3(late) with that for O_3(early) revealed that the difference between them was not statistically significant. The difference between O_3(late) and O_3(mid) was significant, the latter frequency being smaller. This perhaps resulted from 50 percent fewer cells being available for examination for O_3(mid). The data suggested, however, that the observed ozone-induced break frequency did not diminish with time for at least 2 weeks post-exposure.

Animals Exposed to Combined Ozone and Radiation Environment

The exposure-adjusted break frequency for the ozone plus radiation treatment groups, based on the radiation exposure only, was compared with the frequency for the radiation treatment groups. The difference between them, while not statistically significant, was a value that would occur or be exceeded 18 percent of the time (expecting the O_3 + X frequency to be higher). In other words, there was an 18 percent chance that all the breaks observed for the ozone plus radiation treatment resulted from the radiation alone even though the O_3 + X frequency was nearly 40 percent greater than the X frequency. Similarly, there was a 15 percent chance that all the breaks observed for the ozone plus radiation treatment resulted from the ozone exposure alone even though the O_3 + X frequency was 45 percent greater than the O_3(late) frequency.

Another way of examining the ozone plus radiation treatment data is presented in Table 5. The expected breaks from radiation (computed from the exposure-adjusted radiation frequency) and the expected breaks from ozone (computed from the exposure-adjusted ozone frequency) were compared with the number of breaks observed with combined treatment. These facts were revealed:

1. The expected contributions from ozone and from radiation were nearly equal;
2. The number of breaks observed was >70 percent of the total breaks expected from the two agents combined (assuming additive effects);
3. The number of breaks observed was 40 percent greater than the number expected from either agent alone;
4. Either ozone or radiation alone could account for all the breaks seen (considering maximum rather than average expected values).

The data suggested, but did not show conclusively, that the combined ozone plus radiation treatment resulted in a higher frequency of chromosome breaks than would be expected from radiation or from ozone alone.

TABLE 5

COMPARISON OF EXPECTED AND OBSERVED NUMBERS OF BREAKS FOR COMBINED OZONE PLUS RADIATION EXPOSURES

Group	Expected Breaks From Radiation		Expected Breaks From Ozone		Total Expected Breaks[b]		Observed Breaks
	Average	(Maximum)[a]	Average	(Maximum)[a]	Average	(Maximum)	
3	6.6	(9.6)	6.3	(9.4)	12.9	(19.0)	10
4	5.0	(7.3)	4.8	(7.2)	9.8	(14.5)	6
total	11.6	(16.9)	11.1	(16.6)	22.7	(33.5)	16

[a] From upper value of 95 percent confidence interval about the average.

[b] Assuming additive effect of ozone plus radiation.

CHAPTER V

DISCUSSION

The initial estimates of break frequency for radiation and for ozone presented in Chapter II were based on the frequencies expected for deletions and for dicentrics and rings. The observed exposure-adjusted aberration frequencies were compared with these first estimates. These data are presented in Table 6. The $\frac{\text{Observed}}{\text{Estimated}}$ column facilitated comparison, among the four frequencies, of success in estimating.

Expected vs. Observed Aberration Frequencies with Radiation

The estimates for radiation were based on frequencies developed by the irradiation of freshly drawn human whole blood *in vitro* with culturing following immediately (155). These frequencies were found to be reasonably good estimates of *in vivo* aberration production in humans (165), generally within a factor of two for deletions and a factor of six for dicentrics and rings. Frequencies for aberration production in lymphocytes of other species, either *in vitro* or *in vivo*, were not available for comparison.

Data were available for the production of breaks in other Chinese hamster cells by radiation. Exposure-adjusted frequencies for the production of chromatid-type breaks in bone marrow cells with *in vivo* exposure and in cultured "fibroblast-like" (embryo) cells with *in vitro* exposure were nearly equal to one another at $5 \times 10^{-3} \frac{\text{breaks}}{\text{cell-R}}$. For the bone marrow cells, the frequency decreased exponentially with time after

TABLE 6

COMPARISON OF ESTIMATED AND OBSERVED EXPOSURE-ADJUSTED ABERRATION FREQUENCIES

Agent	Exposure-Adjusted Aberration Frequency	Estimated Coefficient	Observed Coefficient Average[a]	Observed Coefficient (Sample Standard Deviation)	$\frac{\text{Observed}}{\text{Estimated}} \times 10^2$
Radiation	$\frac{\text{deletions}}{\text{cell-rad}} \times 10^3$	1	0.275[b]	(0.0872)	27.5
	$\frac{\text{dicentrics + rings}}{\text{cell-rad}^2} \times 10^6$	6	0.687[b]	(0.308)	11.5
Ozone	$\frac{\text{deletions}}{\text{cell-(ppm-min)}} \times 10^3$	3	1.28[c]	(0.358)	42.7
	$\frac{\text{dicentrics + rings}}{\text{cell-(ppm-min)}^2} \times 10^6$	7	2.48[c]	(1.79)	35.4

[a] Treatment average weighted by number of useable spreads per group.

[b] From X groups.

[c] From O_3(late) groups.

irradiation to $1 \times 10^{-4} \frac{\text{breaks}}{\text{cell-R}}$ at 2 days. The initial frequency was approximately a factor of nine greater than the chromosome-type break frequency determined in this investigation (172). The dissimilarity does not seem unreasonable considering the differences in cell type and stage of division at irradiation (86, 148).

Frequencies involving radiation that were obtained in this investigation were for cells withdrawn approximately 2 weeks after exposure. The delay before successful cultures could be obtained, perhaps related to mitotic inhibition by irradiation (86), was not observed in the human in vivo investigations (155, 165), but the doses were considerably lower, 100 rad or less. In the human studies, the aberration levels remained relatively constant for 3-4 weeks after irradiation (155). The observation does not necessarily apply to this investigation, for the turnover times of the two lymphocyte populations may not be the same. The 2 week frequency for Chinese hamsters would undoubtedly be less than or equal to the no-delay frequency (if it could be determined employing a lower dose).

Considering all these factors, the aberration frequencies for radiation determined in this investigation appear reasonable in comparison to available data. They provide information that is basic to additional studies of the peripheral lymphocyte technique as a biological dosimeter.

Chromosome Aberrations and In Vivo Ozone Exposure

Referring to Table 6, the observed exposure-adjusted aberration frequencies for ozone were surprisingly close to the initial estimates considering the numerous assumptions that were involved in computing the expected frequencies (See Chapter II). Besides the data that were used for the initial estimates, from in vitro exposure of cultured human cells to high concentrations of ozone, there were no frequencies for comparison. This was the first investigation of chromosome aberrations and inhaled ozone to be reported. The results add weight to the arguments for considering ozone as a radiomimetic agent.

It was instructive to compare the break frequencies that would be expected from one week of exposure to radiation and to ozone at the maximum permissible industrial exposure levels. The exposure-adjusted break frequencies determined in this investigation were presumed to apply directly to human exposure (thus being conservative for radiation). In one week the permitted average radiation exposure of 100 mrad would result in a lymphocyte chromosome break frequency of 5.5×10^{-5} breaks/cell. One week (40 hr) at the permitted 0.1 ppm for ozone would result in 0.4 breaks/cell!

Certainly the extension of animal data directly to humans could have introduced considerable error in the ozone estimate, but the difference in these two frequencies is too great to be easily explained away. These data do reflect the fact that the limit for radiation recognized its mutagenic ability while the limit for ozone did not. The ozone concentration was set at a value chosen to prevent the occurrence of physical symptoms in most industrially exposed people, namely, nose and throat irritation (84). The recent "Community Air Quality Guide" (192) issued for ozone by the American Industrial Hygiene Association referred to the radiomimetic nature of ozone in stating that "theoretically, the recommended air limit for ozone . . . should be zero, or as close to zero as possible, i.e., less than 0.01 ppm." As a realistic limit, the Guide recommended an upper concentration limit of 0.05 ppm and an exposure limit of 0.1 ppm-hr/day on the average during any year "if human health is not to be significantly impaired during a lifetime of exposure." Projecting again, this level would produce 1,270x more lymphocyte chromosome breaks than the maximum permitted occupational radiation exposure. The data from this limited investigation suggest that a lowering of the community and industrial limits for ozone would be prudent.

Interactions Resulting from Simultaneous Ozone and Radiation Exposure

The data suggested, but did not show conclusively, that the frequency of chromosome aberrations in circulating lymphocytes resulting from either radiation or ozone exposure would increase if exposure to the other agent occurred at the same time. The combined effect, however, would be less than the sum of the effects expected for each agent alone. Some mode of antagonistic action or protective mechanism is therefore suggested, but its nature is not apparent (See Chapter I). The importance of combined effects is overshadowed by the magnitude of the effect expected for ozone alone at permitted concentrations.

CHAPTER VI

CONCLUSIONS

In this investigation, Chinese hamsters were exposed to x-radiation, to ozone, or to both simultaneously. The basic radiation dose was 230 rad delivered in 5 hr. The average ozone exposure was 5 hr at a concentration of 0.2 ppm. Exposure-adjusted frequencies of chromosome aberrations produced in circulating blood lymphocytes served as the quantitative measure of widespread damage. The basic purpose of the study was to provide information on the combined effect of exposure to these agents.

Summary of Results

The following were the principal results in this investigation.

1. Radiation exposure resulted in an exposure-adjusted break frequency $5.51 \times 10^{-4} \frac{\text{breaks}}{\text{cell-rad}}$ for cells withdrawn 2 weeks after exposure. This value appeared reasonable in comparison to available information on *in vivo* exposure of human lymphocytes and Chinese hamster bone marrow cells. Successful lymphocyte cultures could not be obtained until 2 weeks had elapsed. The number of breaks per cell appeared to vary linearly with dose in the region 230-330 rad.

2. The action of inhaled ozone resulted in chromosome aberrations. The exposure-adjusted break frequency was 1.67×10^{-3} $\frac{\text{breaks}}{\text{cell-(ppm-min)}}$. This value was in good agreement with the

expected value calculated on the basis of in vitro exposure of human cells. There was no apparent decrease in break frequency with time for 2 weeks post-treatment.

3. Animals exposed to the two agents simultaneously exhibited >70 percent of the total number of breaks anticipated assuming the actions of the agents to be additive. The expected contributions from ozone and from radiation were nearly equal. There was, however, approximately an 18 percent chance that all the breaks observed resulted from the radiation exposure alone. Similarly, there was a 15 percent chance that all the breaks observed resulted from the ozone exposure alone.

Significance of Results

This experiment demonstrated the suitability of lymphocyte chromosome breakage as a biological indicator of exposure to an air pollutant, ozone. The technique, introduced by this study, may be useful for other oxidant pollutants too. Additionally, the investigation provided data on the in vivo production of lymphocyte chromosome aberrations in an animal by external ionizing radiation; such information was not readily available in the literature. The work demonstrated still another reason for considering ozone as radiomimetic.

Chinese hamsters appeared to be excellent laboratory animals for this type of study when used with the orbital bleeding technique and the lymphocyte culture method which was developed during the investigation. This report presented the first detailed description of a successful method for the short-term culture of circulating blood lymphocytes from Chinese hamsters using moderate size blood samples.

The most important single fact to emerge from this investigation was that inhaled ozone resulted in chromosome aberrations in circulating blood lymphocytes. Because of the body-wide effects of inhaled ozone and the fact that production of chromosome aberrations by ozone appeared to be an action that was general to all cell types, the lymphocyte result should be indicative, in a qualitative sense, of similar damage to other cells throughout the entire body. Such aberrations are considered as cellular alterations (damage) with long-term consequences.

Presently permitted human ozone exposures would be expected to result in break frequencies that are orders of magnitude greater than those resulting from permitted human radiation exposures if the results of this animal study were directly extended to the human case. Consideration of combined ozone plus radiation environments is overshadowed by the importance of ozone environments alone as long as permitted ozone exposure levels remain at their present and seemingly high values.

That ozone inhalation does result in chromosome aberrations at the apparent frequencies is an outcome of this limited investigation which certainly appears worthy of further experimental consideration. In fact, such investigation appears mandatory!

APPENDICES

APPENDIX 1

PREPARATION OF CULTURE MEDIA AND SOLUTIONS

Heparin Solution for Blood Pipettes

To prepare 20 ml of sterile normal (0.85 percent) saline solution with 10^3 USP units heparin/ml, do the following in order:

1. Place 0.153 g of desiccator-dried sodium chloride (NaCl) in a glass screw-cap bottle marked for 20 ml;
2. Add approximately 15 ml of distilled demineralized water;
3. Autoclave at 121.5°C, 15 psig for 15 min;
4. Add sterile distilled demineralized water to bring the volume to 18 ml;
5. Add 2 ml of 10^4 USP units/ml isotonic aqueous sodium heparin solution[13] and mix.

Steps 4 and 5 must be done with sterile technique. Keep the solution refrigerated.

Hungerford Culture Media

To prepare a 115 ml batch of media, add the components listed in Table 7 (180) to a sterile glass screw-cap bottle, aseptically and in sequence.[14] Keep the final solution refrigerated.

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[13] Organon, Inc., West Orange, New Jersey.

[14] This sequence was suggested in Price and Reference Manual, Grand Island Biological Company, Grand Island, New York (1969).

TABLE 7

INGREDIENTS FOR SPECIAL CULTURE MEDIA

Component[a]	Quantity, ml
1. Sterile distilled demineralized water	78
2. Earle's balanced salt solution (10x)	10
3. MEM vitamins (100x)	2[b]
4. MEM essential amino acids (50x)	4[b]
5. L-glutamine, 200 mM (100x)	1
6. Phenol red solution, 0.5%	0.14
7. Sodium bicarbonate solution, 7.5%	3-5[c]
8. Penicillin-streptomycin mix, 5,000 units each/ml	2
9. Fetal bovine serum (gamma-globulin free)	15
10. Aqueous isotonic sodium heparin solution, 10^4 USP units/ml[d]	0.2

[a] Except for 1 and 10, all materials were obtained from Microbiological Associates, Inc., Bethesda, Maryland.

[b] This quantity produced a solution that was twice the normal concentration for this component.

[c] Sufficient sodium bicarbonate was used to produce an orange-amber color at 4°C (pH 7.3).

[d] Organon, Inc., West Orange, New Jersey.

Mast Ozone Meter Sensing Solution

To prepare 1 l. of KI sensing solution (184), do the following in order, using reagent grade chemicals:

1. Add 20 g potassium iodide (KI) to approximately 500 ml of distilled demineralized water in a 1 l. beaker;
2. Add 50 g potassium bromide (KBr); stir to dissolve;
3. Add 2.5 g of sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$);
4. Add 7.0 g of sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7\ H_2O$);
5. Transfer the solution to a 1 l. volumetric flask and bring to 1 l. with distilled demineralized water.

Keep the solution well stoppered.

TC-199 Culture Media with Heparin

To prepare a 100 ml batch of media, add the components listed in Table 8 to a sterile glass screw-cap bottle, aseptically and in sequence. Keep the final solution refrigerated.

TABLE 8

INGREDIENTS FOR TC-199 CULTURE MEDIA WITH HEPARIN

Component	Quantity, ml
1. Medium 199 without $NaHCO_3$ (10x)[a]	10
2. Sterile distilled demineralized water	90
3. Sodium bicarbonate solution, 7.5%[a]	0.47[b]
4. Aqueous isotonic sodium heparin solution, 10^4 USP units/ml[c]	0.2

[a] Microbiological Associate, Inc., Bethesda, Maryland.

[b] The 10x TC-199 concentrate has phenol red. The $NaHCO_3$ brings the solution to pH 7.4 (bright orange color).

[c] Organon, Inc., West Orange, New Jersey.

Colchicine Solution for Mitotic Arrest

The preparation of the working solution which is 2 μg colchicine/ml of normal (1 percent) buffered saline is done in three steps:

Preparation of Normal Buffered Saline[15]

To prepare 10 ml of sterile normal (1 percent) buffered saline solution, do the following in order:

1. Place 0.90 g of desiccator-dried sodium chloride (NaCl) in a glass screw-cap bottle marked for 100 ml;

2. Add approximately 60 ml of distilled demineralized water;

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[15] This is a modification of the procedure suggested by George Cartwright in Diagnostic Laboratory Hematology, 4th edition, Grune and Stratton, New York (1968).

3. Add 0.137 g of anhydrous sodium phosphate, dibasic (Na_2HPO_4);

4. Add 0.021 g of sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$);

5. Autoclave at 121.5°C, 15 psig for 15 min;

6. Add sterile distilled demineralized water to bring the volume to 100 ml.

Step 6 must be done with sterile technique. Keep the solution refrigerated. The pH should be 7.4.

Preparation of Colchicine Stock Solution

To prepare 10 ml of normal saline solution which has a colchicine concentration of 1 mg/ml, transfer 10 ml of the normal buffered saline solution to a sterile screw-cap bottle and add 10 mg of USP colchicine[16] ($C_{22}H_{25}NO_6$), using sterile technique. Keep the solution refrigerated.

Preparation of Colchicine Working Solution

To prepare 50 ml of normal saline solution which has a colchicine concentration of 2 μg/ml, transfer 50 ml of the normal buffered saline to a sterile screw-cap bottle and add 0.1 ml of the colchicine stock solution, using sterile technique. Keep the solution refrigerated.

Hypotonic KCl Solution for Cell Swelling

To prepare a 400 ml batch of 0.075 M potassium chloride (KCl) solution with 16 USP units heparin/ml, do the following in order:

1. Add 2.24 g KCl (reagent grade) to approximately 200 ml of distilled demineralized water in a screw-cap bottle marked for 400 ml;

2. Autoclave at 121.5°C, 15 psig for 15 min;

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[16] Calbiochem, Los Angeles, California.

3. Add sterile distilled demineralized water to bring the volume to 400 ml;

4. Add 0.64 ml of 10^4 USP units/ml istonic aqueous heparin solution[17] and mix.

Steps 3 and 4 must be done with sterile technique. Keep the solution refrigerated.

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[17] Organon, Inc., West Orange, New Jersey.

APPENDIX 2

ALKALINE KI METHOD FOR OZONE DETERMINATION

The following method for ozone determination was taken from Selected Methods for the Measurement of Air Pollutants, a 1965 U. S. Public Health Service publication (185).

Introduction

This method is intended for the manual determination of oxidants (including ozone) in the range of a few parts per hundred million (pphm) to about 20 ppm. Ozone, chlorine, hydrogen peroxide, organic peroxides, and various other oxidants will liberate iodine by this method. The response to nitrogen dioxide is limited to 10 percent by the use of sulfamic acid in the procedure to destroy nitrite. It is customary for convenience to express the results as ozone. The advantage of this procedure over the neutral iodide procedures is that a delay is permissible between sampling and completion of the analysis. Sampling is conducted in midget impingers containing 1 percent potassium iodide in 1 N sodium hydroxide. A stable product is formed that can be stored with little loss for several days. The analysis is completed in a laboratory by addition of phosphoric-sulfamic acid reagent, which liberates the iodine. The yellow iodine color is read in an appropriate instrument. Serious interfering effects occur from reducing gases and dusts.

Reagents

All reagents are made from analytical-grade chemicals. They are stable for several months in well-stoppered bottles.

Double-Distilled Water, Used for All Reagents

Redistill distilled water in an all-glass still after adding a crystal each of potassium permanganate and barium hydroxide.

Absorbing Reagent

Dissolve 40.0 g of sodium hydroxide in almost a liter of water, then dissolve 10.0 g of potassium iodide and make the mixture to 1 l. Store in a glass bottle with a screw-cap (with inert liner) or rubber stopper (previously boiled for 30 min in alkali and washed). Age for at least 1 day before using.

Acidifying Reagent

Five g of sulfamic acid is dissolved in 100 ml of water, then 84 ml of 85 percent phosphoric acid is added, and the mixture is made to 200 ml.

Standard Potassium Iodate Solution

Dissolve 0.758 g of potassium iodate in water and dilute to 1 l. One ml of this stock solution is equivalent to 400 μl of ozone. Prepare a dilute standard solution just before it is required by pipetting exactly 5 ml of stock solution into a 50 ml volumetric flask and making to mark with water.

Apparatus

Absorber

All-glass midget impingers with a graduation mark at 10 ml are used. (Other bubblers with nozzle or open-end inlet tubes may be used. Fritted bubblers tend to give relatively low results.) Impingers must be kept scrupulously clean and dust free. All traces of grease should be removed by treatment with dichromate-concentrated sulfuric acid solution (cleaning solution) followed by tap and distilled water.

Air-Metering Device

A glass rotameter capable of measuring a flow of 1-2 l./min with an accuracy of ± 2 percent is recommended.

Air Pump

An appropriate suction pump capable of drawing the required sample flow for intervals of up to 30 min is suitable. It is desirable to have a trap on the inlet to protect the needle valve and pump against accidental flooding with absorbing reagent and consequent corrosion.

Spectrophotometer

A laboratory instrument suitable for measuring the yellow color at 352 mμ with stoppered tubes or cuvettes (suitable for ultraviolet use) is recommended.

Analytical Procedures

Collection of Samples

Assemble a train composed of a midget impinger, rotameter, and pump. Use ground-glass connections up-stream from the impinger. Butt-to-butt glass connections with slightly greased tygon tubing may also be used for connections without losses if lengths are kept minimal. Pipet exactly 10 ml of the absorbing solution into the midget impinger and sample at a flow rate of 1-2 l./min. Note the total volume of the air sample. If the sample air temperature and pressure deviate greatly from 25°C and 760 mm Hg, measure and record these values. Sufficient air should be sampled so that the equivalent of 1-15 μl of ozone is absorbed. If appreciable evaporation has occurred, add distilled water to restore the volume to the 10 ml graduation mark. If the analysis is to be completed later, transfer the solution, without rinsing, to a clean, dry, glass-stoppered, 25 ml, graduated cylinder (or a bottle) for storage. Prolonged storage may cause "freezing" of glass stoppers.

Analysis

Measure the volume of exposed absorbing reagent in a 25 ml, glass-stoppered, graduated cylinder. (Do not use rinse water in any transfers.) Add from a rapid (serological-type), graduated pipette exactly one-fifth of this volume of the acidifying reagent and mix thoroughly. Place the stoppered cylinder in a water bath at room temperature for 5-10 min to dissipate the heat of neutralization. Transfer a portion of the sample to a cuvette and determine the absorbance at 352 mμ with a cuvette containing distilled water as the reference. Do not delay the reading, since reducing impurities sometimes cause rapid fading of the color.

Prepare a reagent blank by adding 2 ml of the acidifying reagent to 10 ml of unexposed absorbing reagent. Cool and determine the blank absorbance. This blank absorbance should be determined each day and should be subtracted from the absorbances of the samples.

Samples may be aliquoted either before or after acidification if very large concentrations of oxidant are expected. In the former case, dilute the aliquot to 10 ml with unexposed absorbing reagent and proceed in the usual manner. In the latter case, dilute the aliquot to volume with reagent blank mixture. Aliquoting after acidification is not as reliable and should be used only to save a sample when unexpectedly large concentrations of oxidant are encountered. The calculations should include the aliquoting factor.

Standardization

Add the freshly prepared, dilute, standard iodate solution in graduated amounts of 0.10-0.45 ml (measured accurately in a graduated pipet or small buret) to a series of 25 ml, glass-stoppered, graduated cylinders. Add sufficient alkaline potassium iodide solution to make the total volume of each exactly 10 ml. Acidify and determine the absorbance of each standard as with the samples.

Calculations

For convenience, standard conditions are taken as 760 mm of mercury and 25°C; thus, usually only slight correction by means of the well-known perfect gas equation is required to get V, defined as the standard volume of the air sampled in liters. Ordinarily this correction may be omitted. Quantities, customarily expressed in terms of ozone, may be expressed as microliters, defined as V x ppm ozone. It has been determined empirically that 1 mole of ozone liberates 0.65 mole of iodine (I_2) by this procedure. The strength of the stock standard potassium iodate solution is computed on the basis of $I_2 \rightleftharpoons 1/3\ KIO_3$, and the following:

Standard molar volume (25°C, 760 mm) = 24.47 liters;

molecular weight $KIO_3 = 214.02$; 1 ml stock solution = 400 μl

$$(O_3) = \frac{400 \times 10^{-6}}{24.47} \times \frac{0.65}{3} \times 214.02 = 0.758 \times 10^{-3} \text{ g } KIO_3$$

Plot the absorbances of the standards (corrected for the blank) against milliliters of dilute standard iodate solution. Beer's Law is followed. Draw the straight line giving the best fit and determine the value in milliliters of the diluted potassium iodate solution intercepted at an absorbance at exactly 1. This value multiplied by 40 gives the standardization factor M, defined as the number of microliters of ozone required by 10 ml of absorbing reagent to give a final absorbance of 1. For 2 cm cells this value is 9.13. Then:

$$\text{ppm oxidant (expressed as } O_3) = \text{corrected absorbance} \times \frac{M}{V}$$

If the volume of the air sample, V, is a simple multiple of M, calculations are simplified. Thus, for the M value of 9.13 previously cited, if exactly 9.13 l. of air is sampled through the impinger, the corrected absorbance is also ppm directly. If other volumes of absorbing reagent are used, V is taken as the volume of air sample per 10 ml of absorbing reagent.

Discussion of Procedure

Sampling Efficiency

When two impingers are placed in series, iodine will very rarely be liberated from the solution in the second absorber. Thus, the sampling efficiency is very high. Fritted bubblers, which also appear to have equally high sampling efficiences, usually give, however, less iodine for a given amount of ozone and should not, therefore, be used. (This is due to the complex chemistry of ozone in alkaline iodide solution. It appears that hypoiodite is the primary product, but that some is lost through side reactions, with resulting variation in the stoichiometry. Potassium iodate is a convenient chemical to use for standardization, although iodate is probably not produced by adsorption of ozone.)

Stability of Exposed Absorbing Reagent

Studies have indicated that most of the losses in exposed reagent occur in the first day. The reagent may then be stored for as long as a week or more with little further change. Use of analytical-grade reagents and of carefully cleaned glassware reduces losses.

Acidification Step

Certain irreversible losses of microquantities of iodine occur during the acidification of alkaline iodide solution. This is probably the explanation for the relationship of 0.65 mole of iodine liberated for each mole of ozone absorbed. Very slow acidification will yield less iodine. The most reproducible procedure is to add the acid rapidly with vigorous stirring.

Interferences

The negative interferences from reducing gases such as sulfur dioxide and hydrogen sulfide are very serious (probably on a mole-to-mole equivalency). The procedure is very sensitive to reducing dusts that may be present in the air or on the glassware. Losses of iodine also occur even on clean glass surfaces, and thus the manipulations should minimize this exposure.

APPENDIX 3

PROCEDURE FOR STAINING SLIDES

The following procedure[18] was employed for staining the Chinese hamster lymphocyte culture slides with Giemsa[19] blood stain:

1. Prepare the stain solution by mixing 3 parts concentrated Giemsa solution with 5 parts distilled water; mix thoroughly;
2. Place the slide into the liquids listed in Table 9, in sequence, for the times indicated.

TABLE 9

SLIDE STAINING LIQUIDS AND REQUIRED TIMES

Liquid	Time Required
1. Stain solution	20 min
2. Distilled water	quick rinse
3. Distilled water	quick rinse
4. Acetone	15 sec with agitation
5. Toluene	3 min
6. Xylene	3 min

3. While the slides are still wetted with xylene, cover them with glass slips fastened with Permount.[19]

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[18] This procedure was used for staining human lymphocyte culture slides in the laboratory of Dr. Gerald Bloom, Department of Pediatrics, University of Florida, Gainesville.

[19] Fisher Scientific Company, Springfield, New Jersey.

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BIOGRAPHICAL SKETCH

Ronald Edward Zelac was born January 22, 1941, in Chicago, Illinois. He attended the University of Illinois as a Cook County Scholar and was granted the degree of Bachelor of Science in Engineering Physics with highest honors in 1962. While pursuing his undergraduate work, he was recognized by being awarded University Honors and the College of Engineering Bell Award as well as by initiation into Phi Eta Sigma, Tau Beta Pi, and Phi Kappa Phi honor societies. He was one of ten in the nation to receive a Phi Kappa Phi fellowship for graduate study in 1962. Granted the Master of Science degree in Physics from the University of Illinois in 1964, he then continued his graduate work at the University of Michigan under the U. S. Atomic Energy Commission fellowship program and was awarded the Master of Science degree in Environmental Health Sciences in 1965.

His work experience has been as follows: Argonne National Laboratory, Student Aide, 1962; Coordinated Science Laboratory, U. of Illinois, Research Assistant, 1963-1964; Argonne National Laboratory, Health Physics Trainee, 1965; IIT Research Institute, Chicago, Chief Health Physicist, 1965-1968; and Mercy Hospital Radiation Therapy Center, Chicago, Radiation Physicist, 1967-1968. He has been affiliated with the Health Physics Society, locally and nationally, since 1967, the American Association of Physicists in Medicine, Midwest Chapter, and the American Industrial Hygiene Association, Chicago Section. He has writings in the area of radiological health that have been published in the open literature and distributed as research reports.

His work toward the degree of Doctor of Philosophy at the University of Florida, as a U. S. Public Health Service Trainee, began in March, 1968. Upon completion of the requirements for this degree, he will assume the position of Radiation Safety Officer and Assistant Professor of Bionucleonics at Temple University, Philadelphia, Pennsylvania.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Engineering and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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